Human Immunodeficiency Virus-1 Induces Loss of Contact Inhibition in Podocytes

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Abstract. Human immunodeficiency virus–associated nephropathy (HIVAN) affects up to 10% of HIV-positive black adults and children and is the leading cause of renal disease in infected individuals. The disease is characterized by proliferation of renal epithelial cells, both glomerular and tubular. Diseased kidneys are enlarged, and glomerular visceral epithelial cells (podocytes) express proliferation markers. In a transgenic murine model of HIVAN expressing a deletion construct of HIV-1, the identical pathologic features are observed. It was demonstrated that HIV-1 mRNA is expressed in renal epithelium of the transgenic mouse and in patients with HIVAN, suggesting a direct role for HIV-1 in disease pathogenesis in both humans and the murine model. For investigating the mechanisms responsible for proliferative changes in podocytes, the HIV-1 transgenic mouse was bred onto the immunocompetent murine background, and conditionally immortalized transgenic and nontransgenic podocyte cell lines were established. Transgenic podocytes demonstrated increased spontaneous proliferation, compared with nontransgenic podocytes at confluence, and they were found to have a greater percentage of cells in the proliferative phase of the cell cycle. It is striking that transgenic podocytes were not contact inhibited and formed aggregates in soft agar. Aggregates also formed when nontransgenic podocytes were infected with the identical HIV-1 construct used to generate the transgenic model. This demonstrates that the loss of contact inhibition is due to a direct effect of HIV-1. Therefore, proliferation induced by HIV-1 gene expression is likely to play a key role in the pathogenesis of HIVAN.

Human immunodeficiency virus–associated nephropathy (HIVAN) is the third leading cause of renal disease in black individuals between the ages of 20 and 64 yr. After the onset of end-stage disease, the median survival for patients on dialysis is only 4.5 mo (1). Patients with HIVAN have proteinuria as well as enlarged echogenic kidneys (2–4). In the transgenic mouse model, kidney size is greater, kidneys weigh more, and protein and DNA content are increased when compared with normal kidneys (5). Histopathologic manifestations of HIVAN include tubular dilation and focal and segmental glomerulosclerosis, often with collapse of the glomerular tuft (6). Proteinaceous casts, increased matrix deposition, and protein reabsorption droplets are seen as well. Evidence of proliferation (7) and apoptosis (8) of renal epithelial cells has been noted. The mechanisms of HIVAN pathogenesis, however, remain to be elucidated.

Studies with HIV-1 transgenic murine models have been critical to our understanding of HIVAN (9–11). We previously reported the establishment of a murine transgenic line in which a gag/pol deletion construct of HIV-1 provirus pNL4-3 was used as the transgene under the control of the viral LTR. Env and regulatory genes are expressed, but no infectious virus is produced. Yet renal disease develops in these mice identical to HIVAN in humans. HIV-1 mRNA is expressed in the exact sites of injury in HIVAN in both human and mouse, namely renal glomerular and tubular epithelium (12,13). Markers for proliferation have been localized to these cells as well (7,14). A causal role for HIV-1 in inducing proliferation of epithelial cells has been difficult to establish.

The renal epithelial cell, in particular the glomerular visceral epithelial cell, or podocyte, has emerged as the glomerular target of HIV-1 pathogenesis. Podocytes are terminally differentiated, highly specialized renal cells that function in the regulation of glomerular filtration. Podocyte injury is thought to play a central role in the development of focal segmental glomerulosclerosis (15), particularly the collapsing variant (16). In idiopathic collapsing glomerulopathy and HIVAN, podocytes are thought to proliferate, but in one report the hyperplastic cells are hypothesized to originate from parietal epithelial cells (17). Observed changes in podocyte structural features, the loss of differentiation markers, and the increase in proliferation markers in HIVAN patients suggest that dysregulation of the podocyte leads directly to the development of HIVAN (7). Identical alterations have been found in the trans-
genic murine model, including increased markers of proliferation and loss of markers of differentiation (14). The current hypothesis, then, suggests that HIV-1 gene expression in podocytes results in the deterioration of nephron architecture and loss of glomerular structure and function.

Evidence suggests that expression of viral gene products may play a central role in HIVAN pathogenesis, but no in vitro culture system has been established to permit a detailed investigation of HIV-1 gene expression in podocytes and the potential mechanism by which viral gene expression induces disease. The aim of the present study was to establish such an in vitro culture system and to determine whether HIV-1 gene expression directly causes podocyte proliferation. We found that HIV-1 gene expression induces podocyte proliferation and causes a loss of contact inhibition. The results presented here suggest that HIV-1 gene expression is causative in the podocyte proliferation seen in HIVAN in both humans and transgenic mice.

Materials and Methods

Establishment of Conditionally Immortalized Podocyte Clones

Heterozygous HIV-1 transgenic mice (“Tg26,” FVB/N, pNL4-3 d1443 [– gag, – pol] described previously (12,18–20) were bred with H-2Kb-tsA58 Immortomice (Charles River Laboratories, Wilmington, MA). F1 progeny were tested for the presence of the HIV-1 transgene by Southern blot analysis of genomic DNA. Murine podocytes were isolated from HIV-1 transgenic mice and nontransgenic littermates at the onset of detectable disease in the HIV-1 transgenic mice (3). The current murine model, including increased markers of proliferative (S/G2/M) and resting (G0/G1) phases of the cell cycle was determined. Cells with subdiploid DNA content were considered apoptotic.

Reverse Transcription and PCR

To confirm HIV-1 gene expression in podocyte clones, we extracted total cellular RNA with TRIZOL Reagent (Life Technologies BRL, Grand Island, NY) from HIV-1 transgenic and nontransgenic clonal cultures grown to 90 to 100% confluence at 33°C according to manufacturer’s protocol. Extracted RNA was DNase-treated (Life Technologies BRL) and stored at −80°C until use. Alternatively, total cellular RNA was isolated by use of the RNase-Free DNase Set (QIAGEN, Valencia, CA) as per manufacturer’s protocols. RNA (5 μg) was reverse-transcribed with random primers by use of the Reverse Transcription System (Promega). One fourth of the first-strand cDNA synthesis reaction product was used as template for PCR amplification by use of AmpliTaq DNA Polymerase (Perkin Elmer, Branchburg, NJ) and 5 ng/μl each of HIV-1 env-specific primers QS-Env-2s (TGT CCA AAG GTA TCC TTT GAG CCA ATT CC) and QS-Env-2as (AGT AGA AAA ATT CCC CTC CAC AAT TAA). Mouse G3PDH primers (Clontech Laboratories, Palo Alto, CA) were used as positive controls. After a 3-min hot start at 94°C, 40 cycles of amplification were carried out with the following parameters: denaturation at 94°C for 1 min, annealing at 62°C for 1 min, and synthesis at 72°C for 30 s, with a 5-s increment extension per cycle. A 7-min extension at 72°C was included at the end of the last cycle. PCR products were resolved on a 1% agarose gel and visualized with ethidium bromide staining.

Cell Growth Assays

HIV-1 transgenic and nontransgenic podocytes were plated in 96-well flat-bottom microtiter plates at various densities (1000, 10,000, 50,000, or 100,000 cells/100 μl) and assayed for [3H] thymidine incorporation on day 3 or day 7 after 18 h of incubation. Cell growth was also quantified on day 7 by use of the CellTititer 96 Cell Proliferation Assay (Promega, Madison, WI) according to manufacturer’s instructions and by hemocytometric counting after trypan blue dye exclusion. To determine cell doubling times, we seeded 500 cells of each clone in 96-well plates with 100 μl of medium and allowed them to grow for 14 d. Triplicate wells were trypsinized and counted after trypan blue dye exclusion at 24-h intervals. All growth rate studies were carried out in complete medium supplemented with rIFN-γ, at 33°C, to allow proliferation.

Preparation of Conditioned Medium

HIV-1 transgenic cells were plated at high density, and culture supernatant was removed after 36 h. Conditioned medium was then centrifuged for 10 min to remove cellular debris, filtered, and frozen at −80°C until use. For growth studies, 50,000 cells were plated in flat-bottom 96-well culture plates either in conditioned medium with fresh medium in a 1:1, 1:4, or 1:10 dilution or in fresh medium alone (as controls). All media were complete and supplemented with rIFN-γ. On day 4, medium was removed and replenished with freshly prepared conditioned medium in identical concentrations. On day 7, cells were assayed for [3H] thymidine uptake after 18 h of incubation.

Flow Cytometry

HIV-1 transgenic and nontransgenic cells were grown in flasks at 33°C to 90 to 95% confluence. Cells (106) were trypsinized, washed with 1× phosphate-buffered saline and fixed with ice-cold 80% ethanol with gentle agitation. After incubation at −20°C for 30 min, cells were pelleted, resuspended in 1× phosphate-buffered saline, and passed through a 25-G needle to create a single cell suspension. RNase (1 mg/ml) and propidium iodide (400 μg/ml) were added, and cells were incubated for 30 min at 37°C. Cell cycle analysis was performed on a FACSCalibur flow cytometer, and results were analyzed with CellQuest software (Becton Dickinson, Mountain View, CA). Doublet exclusion was performed by pulse shape analysis (height versus area of the fluorescence pulse). The percentage of cells in the proliferative (S/G2/M) and resting (G0/G1) phases of the cell cycle was determined. Cells with subdiploid DNA content were considered apoptotic.

Growth in Soft Agar

HIV-1 transgenic and nontransgenic podocytes were grown in soft agar in 6-cm plates. Cells (10,000 or 100,000) were suspended in 5 ml 0.2% agar (Fisher Scientific, Fair Lawn, NJ) in complete medium with rIFN-γ, overlaid on 5-ml presolidified 0.4% agar in the same medium, and incubated at 33°C for up to 8 wk.
Normal complete medium with rIFN-γ (1 ml) was layered gently over the cultures every 5 d. Results were scored by three independent observers, who counted all planes of view of 10 fields each, 10× power. Viability of cells was ascertained by staining with vital dye 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride for 48 h.

Construction of Lentiviral Vectors and Infection

For production of pseudotyped virus containing the HIV-1 transgene, 293T cells were cotransfected with transcomplementing vector pCMVΔR9.1 (provided by Dr. Didier Trono, University of Geneva, Geneva, Switzerland), HIV-1 gag/pol deletion construct pNL4-3 d1443, and vesicular stomatitis virus G (VSVG) envelope vector pMD.G. For production of pseudotyped control virus expressing only green fluorescent protein (GFP) but no HIV-1 viral transcripts, 293T cells were cotransfected with transcomplementing vector CMVΔR8.2 (provided by Dr. Inder Verma, The Salk Institute for Biological Studies, La Jolla, CA), pHRCMV-GFP (constructed by inserting eGFP plasmid [Clontech Laboratories] into the vector pHRCMV-Lucif [22] between the sites BamHI and Xhol), and pMD.G. VSV G envelope pseudotyping allows highly efficient entry in a wide variety of cell types. Nontransgenic podocytes (60,000) were grown to 50 to 80% confluence in 6-well plates and infected by these lentiviral vectors. Green fluorescence was seen in control-GFP–infected cells after 3 d. Subsequently, cells were suspended in soft agar as described.

Results

Cells

A conditionally immortalized, HIV-1 transgenic podocyte culture system was established to study the effect of HIV-1 gene expression in podocytes, the glomerular target cells in HIVAN (see Materials and Methods section). At low density, both nontransgenic and HIV-1–transgenic cells displayed a similar epithelial cell phenotype at 33°C, forming rounded and spindle-shaped processes (Figure 1, A and B). When the cells reached confluence, however, the morphology of transgenic and nontransgenic clones diverged. Nontransgenic cells formed a flat monolayer, with several detached apoptotic cells (Figure 1C), whereas transgenic cells overgrew the monolayer, forming hillocks and heaps (Figure 1D). Transgenic cells became smaller and rounder and had a more cobblestone appearance.

Figure 1. Morphology of conditionally immortalized murine podocytes. (A) Nontransgenic cells at low density. (B) HIV-1 transgenic cells at low density. (C) Nontransgenic cells at high density. (D) HIV-1 transgenic cells at high density. All cells shown were grown under permissive conditions. Magnifications: ×30 in A and B; ×20 in C and D.
Expression of HIV-1 and Podocyte Markers
To confirm HIV-1 gene expression in podocyte clones, we performed reverse transcription-PCR (RT-PCR) on total cellular RNA extracted from the HIV-1 transgenic clone and the nontransgenic clone grown at subconfluence and at confluence under permissive conditions. Amplified products were resolved on 1% agarose gels and visualized with ethidium bromide staining. Primers were specific for a 541-bp fragment of HIV-1 env (top) and a 983-bp fragment of G3PDH (bottom) as a control. Lane 1, φX174 marker; lane 2, pNL4-3 d1443 DNA positive control (top), G3PDH DNA positive control (bottom); lane 3, negative control (water); lane 4, HIV-1 transgenic clone, at confluence; lane 5, HIV-1 transgenic clone, at subconfluence; lane 6, nontransgenic clone, at confluence; lane 7, nontransgenic clone, at subconfluence.

Cell Growth Rate Studies
A sigmoidal growth curve was observed for both transgenic and nontransgenic podocytes. Both clones grew at comparable rates until they reached confluence on day 8. At confluence, nontransgenic cell growth plateaued as expected. The transgenic clone, however, continued to proliferate (Figure 3). The differences in cell number were not statistically significant before confluence (P > 0.5), but they were significant after confluence (P < 0.001). Before confluence, the mean doubling time for both nontransgenic and transgenic podocytes was 0.7 d. After confluence, however, nontransgenic podocyte doubling time was 8.1 d, whereas transgenic podocyte doubling time was 4.5 d. To explore the apparent differences in growth properties between the transgenic and nontransgenic cells, we performed the following studies at 95 to 100% confluence at 33°C.

Figure 2. Reverse transcription-PCR (RT-PCR) analysis of HIV-1 transgenic podocyte RNA and nontransgenic podocyte RNA extracted from cells grown at subconfluence and at confluence under permissive conditions. Amplified products were resolved on 1% agarose gels and visualized with ethidium bromide staining. Primers were specific for a 541-bp fragment of HIV-1 env (top) and a 983-bp fragment of G3PDH (bottom) as a control. Lane 1, φX174 marker; lane 2, pNL4-3 d1443 DNA positive control (top), G3PDH DNA positive control (bottom); lane 3, negative control (water); lane 4, HIV-1 transgenic clone, at confluence; lane 5, HIV-1 transgenic clone, at subconfluence; lane 6, nontransgenic clone, at confluence; lane 7, nontransgenic clone, at subconfluence.

Figure 3. Total number of nontransgenic (---) and HIV-1 transgenic (○) cells after 14 d under permissive conditions. Cells (500 of each clone) were plated in 96-well plates with 100 μl of medium. Triplicate wells were trypsinated every 24 h and counted after trypan blue dye exclusion. The arrow marks the time point at which cells became confluent (day 8).

Figure 4. [3H] thymidine incorporation by transgenic and nontransgenic podocytes on day 7. After an 18-h incubation with [3H] thymidine, two- to threefold increases in uptake were seen by transgenic podocytes, compared with nontransgenic podocytes. Bars represent mean ± SD of triplicate samples. □ nontransgenic; ■ HIV-1 transgenic. Experiments were repeated three times.
Tritiated Thymidine Incorporation

Incorporation of $[^{3}H]$ thymidine was measured in transgenic and nontransgenic podocytes. Cells were plated at the same density, grown under permissive conditions, and measured for $[^{3}H]$ thymidine uptake after 7 d. Independent of initial cell density, the transgenic clone incorporated more $[^{3}H]$ thymidine, compared with the nontransgenic clone (Figure 4). The increase in $[^{3}H]$ thymidine was two- to threefold. The same results were obtained at 3 or 7 d, at 33°C or 37°C, and with or without collagen-coated plates. No difference was seen in SV-40 T antigen expression with the HIV-1 transgene (data not shown).

Identical results also were seen when podocyte growth patterns were assessed by use of a proliferation assay that measures cellular metabolism and by enumeration of cells after trypan blue dye exclusion (data not shown). Growth patterns seen with the $[^{3}H]$ thymidine incorporation assay and proliferation assay were consistent with those found measuring cell growth rates and doubling times (Figure 3).

Cell Cycle Analysis

Cell counts and $[^{3}H]$ thymidine incorporation measurements do not provide information on the proportion of cells in a culture that are actively progressing through the cell cycle. To determine the percentage of cells in culture that were in the proliferative phase of the cell cycle (S, G2, or M), we performed flow cytometric cell cycle analysis by using propidium iodide staining. As shown in Figure 5, the percentage of HIV-1–transgenic cells in the proliferating phase (39.4%) was more than twice that of nontransgenic cells (18.2%). In addition, the percentage of apoptotic cells in nontransgenic podocytes (1.4%) was seven times that of transgenic podocytes (0.2%). Hence, a greater percentage of transgenic cells were cycling, correlating with the $[^{3}H]$ thymidine incorporation studies. These data indicated that HIV-1–transgenic podocytes proliferate faster than nontransgenic podocytes at confluence in vitro, consistent with in vivo studies that show increased proliferation in both the murine model and humans (14,16).
To determine whether proliferation was due to an intracellular or extracellular effect of HIV-1 gene expression, we performed the following studies. Stimulation of nontransgenic podocyte growth by conditioned medium from transgenic podocytes was tested to explore whether a secreted factor was sufficient for increased growth of transgenic cells. Tritiated thymidine incorporation was measured in nontransgenic cells cultured with conditioned medium from transgenic culture supernatants. Conditioned medium was prepared in 1:1, 1:4, and 1:10 dilutions with fresh medium. As controls, nontransgenic cells were cultured with conditioned medium from nontransgenic cultures in the same dilutions, and transgenic and nontransgenic cells also were cultured with fresh medium alone, as before.

Conditioned medium from transgenic podocytes did not stimulate [3H] thymidine incorporation in nontransgenic cultures at 1:1, 1:4, and 1:10 dilutions (Figure 6). Transgenic cell [3H] thymidine uptake increased three- to fourfold over uptake in nontransgenic cells, consistent with results shown in Figure 4. These data suggest that the accelerated growth by transgenic podocytes was not due to a secreted factor.

Figure 7. Growth in a soft agar matrix. (A) Nontransgenic podocytes after 4 wk. (B) HIV-1 transgenic podocytes after 4 wk. (C) Nontransgenic podocytes infected with VSV G–pseudotyped control virus containing green fluorescent protein after 4.5 wk. (D) Nontransgenic podocytes infected with VSV G–pseudotyped virus containing HIV-1 transgene construct after 4.5 wk. Magnifications: ×4 in A and B; ×10 in C and D.

[3H] Thymidine Incorporation with Conditioned Medium

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Figure 8. RT-PCR analysis of aggregates extracted from infected and uninfected podocytes grown in soft agar. Amplified products were resolved on 1% agarose gels and visualized with ethidium bromide staining. Top, amplification products with 541-bp HIV-1 env primers. Bottom, amplification products with 983-bp G3PDH primers, as a control. Lanes 1 and 7, φX174 marker; lane 2, RNA from aggregates in HIV-1 transgene–infected cultures; lane 3, RNA from isolated single cells in HIV-1 transgene–infected cultures; lane 4, RNA from isolated single cells in control-GFP–infected cultures; lane 5, RNA from aggregates in transgenic cultures; lane 6, RNA from isolated single cells in nontransgenic cultures.
Growth in Soft Agar

The finding that transgenic podocytes continued to grow after reaching confluence suggests that they were not contact inhibited. To test this hypothesis, we grew transgenic and nontransgenic cells in a soft agar matrix, which typically inhibits untransformed cell growth. Although nontransgenic podocytes remained as isolated single cells suspended in soft agar (Figure 7A), transgenic podocytes continued to proliferate, forming aggregates (Figure 7B). Aggregates were first seen in transgenic cultures after 2 wk. The largest differences in aggregate formation between transgenic and nontransgenic cultures were observed after 4 wk. The average number of aggregates per field in transgenic cultures (11.07) was 85 times that in nontransgenic cultures (0.13). The number of cells in a typical aggregate in transgenic cultures was 16 to 32 cells, whereas that in nontransgenic cultures was 4 to 6 cells. Thus, transgenic podocytes were less contact inhibited in soft agar, compared with nontransgenic podocytes.

Confirmation of HIV-1 Gene Expression in Soft Agar

To confirm that the proliferating cells contained the HIV-1 construct, we extracted aggregates from soft agar cultures. RT-PCR was performed on total cellular RNA isolated from the aggregates. As shown in Figure 8, a 541-bp fragment of HIV-1 env was readily amplified from aggregates in HIV-1 transgene–infected cultures and from transgenic aggregates but not from isolated single cells in HIV-1 transgene–infected cultures, control-GFP–infected cultures, or nontransgenic cultures. Cells from all cultures produced a 983-bp signal for the housekeeping gene G3PDH. It is noteworthy that no amplified HIV-1 product was found in single cells isolated from transgene-infected cultures, which suggests that cells uninfected by the VSV-pseudotyped virus contained no HIV-1 env and did not proliferate, even in transgene-infected cultures. The 983-bp G3PDH signal indicated the presence of RNA in this sample.

Discussion

The results presented here are consistent with a model in which HIV-1 gene expression in podocytes results in increased cell proliferation by inducing a loss of contact inhibition. Podocyte proliferation is a prominent feature of HIVAN in vivo, both in humans (16) and in the transgenic murine model (14). An abnormal proliferative response is likely to disrupt glomerular architecture by altering the differentiated phenotype of podocytes that is critical for structural integrity and thus contributes to collapse of the glomerulus (7,14).

To examine whether HIV-1 induces podocyte proliferation, we established a podocyte cell culture system that closely resembles our transgenic mouse model, because it contains the HIV-1 transgenic deletion construct. We found that transgenic cells proliferated at a faster rate than nontransgenic cells at confluence (Figure 3). This difference was not attributable to the role of a labile soluble factor or the requirement for both a soluble factor and cell autonomous effects induced by HIV-1 cannot be excluded. It is notable that cell synchronization was not necessary to see the differences in growth patterns between the transgenic and nontransgenic clones. SV-40 T antigen may play a role in this cell culture system, but it is not sufficient to enhance growth, because the nontransgenic cells (expressing T antigen but not HIV-1 genes) did not proliferate like the HIV-1 transgenic cells. It is striking that transgenic cells formed aggregates in a soft agar matrix, whereas nontransgenic cells did not (Figure 7). This suggests that HIV-1–transgenic cells were not contact inhibited. Furthermore, the nontransgenic podocyte clone infected with the HIV-1 transgene construct also formed aggregates (Figure 7), which confirmed that this effect was the direct result of HIV-1 gene expression and not of clonal variation.

Although direct infection of the kidney by HIV-1 has been debated during recent years, new data provide convincing evidence that HIV-1 replicates in renal epithelium. Using in situ hybridization of renal biopsy tissue from HIVAN patients, Bruggeman et al. (13) showed that HIV-1 mRNA is detectable in renal epithelial cells, including visceral and parietal glomerular epithelial cells and tubular epithelial cells, as well as in interstitial cells. The detection of extrachromosomal circumscribed proviral DNA by PCR, which indicates nuclear import of HIV-1 after recent infection, as well as proviral DNA by in situ PCR, confirms further that the kidney is a target for HIV-1 infection and viral replication in man.
In summary, several mechanisms may contribute to the increased growth of podocytes. The most likely explanation is that single or multiple HIV-1 gene products directly impair cell–cell or cell–extracellular matrix interactions to induce a loss of contact inhibition, which then alters cell cycle progression and results in enhanced proliferation.

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