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bFGF and its low affinity receptors in the pathogenesis of HIV-associated nephropathy in transgenic mice

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bFGF and its low affinity receptors in the pathogenesis of HIV-associated nephropathy in transgenic mice. HIV-associated nephropathy is characterized by extensive tubulointerstitial disease with epithelial cell injury, microcystic proliferation, and tubular regeneration with glomerulosclerosis. To explore the role of bFGF as a mediator of HIV-induced interstitial disease, we utilized an HIV transgenic mouse model that manifests clinical and histological features observed in patients. In transgenic mice, simultaneous renal epithelial cell proliferation and injury were detected *in vivo*. In areas of microcystic proliferation, immunoreactive bFGF colocalized with extracellular matrix. Kidneys from transgenic mice had increased bFGF low affinity binding sites, particularly in the renal interstitium. *In vitro*, transgenic renal tubular epithelial cells proliferated more rapidly and generated tubular structures spontaneously, in marked contrast to nontransgenic renal cells where these pathologic features could be mimicked by exogenous bFGF. These studies suggest that renal bFGF and its receptors play an important role in the pathogenesis of HIV-associated nephropathy.

Enlarged kidneys with histopathological evidence of renal tubular epithelial cell (RTEC) injury, microcystic proliferation, and tubular regeneration are distinctive features of HIV-associated nephropathy (HIVAN). Mesangial hyperplasia, focal segmental and global glomerular sclerosis and reactive hyperplasia of the renal epithelium are observed as well [1–4]. Although the fundamental mechanisms responsible for these proliferative and degenerative changes are unknown, many hypotheses have been proposed; the glomerular or tubular abnormalities characteristic of HIVAN could result from replicating virus in renal cells [5], associated drug toxicity [6, 7], coexistent opportunistic infections [6, 8, 9], and genetic predisposition [10]. In addition, it has been suggested that circulating mediators, including cytokines, viral proteins, or immune complexes [2–8, 11] may act upon renal parenchymal cells to induce the characteristic histological changes of HIVAN.

Recently, we have described HIVAN in mice made transgenic for a subgenomic proviral HIV-1 construct [12, 13]. Transgenic mice experience progressive renal disease that is both histologically and clinically similar to that of patients infected with HIV-1. In these mice, renal disease presents with proteinuria and progresses rapidly to end-stage disease [13]. Kidneys are enlarged and there is histologic evidence of glomerulosclerosis and severe microcystic tubular dilatation [12, 13].

Increasing evidence supports an important role for members of the fibroblast growth factor (FGF) family in proliferative diseases associated with HIV-1 infection. The oncogene hst/K-FGF which encodes FGF-4, a growth factor that is highly mitogenic for fibroblasts, was discovered by transfection of Kaposi's sarcoma DNA into NIH 3T3 cells [14]. Another gene product related to the FGF family int-2 is overexpressed in Kaposi's sarcoma cells and may play a role in stimulating local angiogenesis [15]. Immunoreactive bFGF has been detected in Kaposi's sarcoma lesions in AIDS patients by immunocytochemistry [16, 17]. This member of the FGF family has been clearly shown to be present in renal tissues as well [18–20].

Basic FGF is a highly conserved 18 kDa cationic protein that belongs to the family of heparin binding growth factors [21]. It is primarily a cell-associated protein with important roles in fetal development, neovascularization, wound healing, and neuronal and smooth muscle cell growth [18, 21, 22]. In the fetal mouse kidney, bFGF is localized within the basement membranes surrounding renal tubules [18] and it is mitogenic for renal epithelial cells [23, 24]. These findings suggest an important role for bFGF during renal tubular development. A role in renal pathogenesis, however, remains less clearly defined. bFGF released by injured mesangial cells in a rat model of immunologically-induced glomerulonephritis stimulates the proliferation of mesangial cells [25]. In addition, in diseases characterized by renal epithelial proliferation and injury such as renal and bladder tumors [26–28], increased bFGF can be detected in the urine suggesting that during this process bFGF can be released from cells. Furthermore, the role of bFGF in HIV pathogenesis remains unclear. In the present study, we present evidence to support an important role for bFGF and its receptors in the pathogenesis of HIVAN.

Methods

Transgenic mice

We have introduced into mice as a transgene, a replication defective HIV-1 proviral construct lacking the *gag* and *pol* genes, pNL4-3:d1443 [12, 13]. This construct contains the viral long terminal repeats (LTRs) and encodes envelope, the accessory

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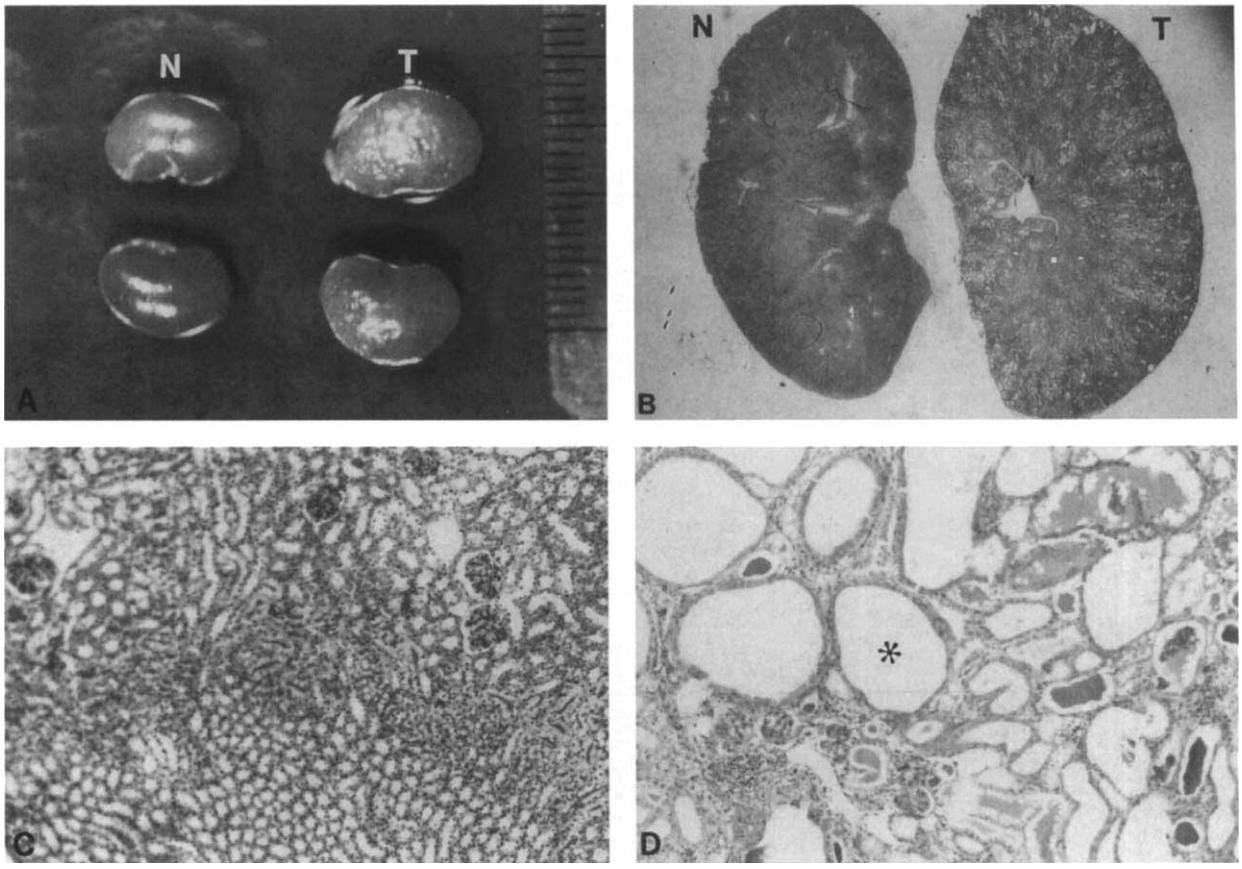


Fig. 1. *Comparison of kidney size and renal histology in control (N) and transgenic (T) mice.* As shown in panels **A** and **B**, transgenic kidneys were enlarged when compared with normal controls. Control and transgenic kidneys are shown at higher magnification (160×) in panels **C** and **D**, respectively. Typical interstitial features of HIV associated nephropathy in man were observed in transgenic mouse kidneys (panel D) with tubular dilatation, microcyst formation (*), and the presence of proteinaceous casts in luminal fluid. Transmission electron micrographs of renal epithelial cells are shown from control (**E**) and transgenic (**F**) mouse kidneys. In normal proximal renal tubular epithelial cells, microvilli (MV), mitochondria (MT), lysosomes (LV), basal infolding of cell membranes (BI), basal lamina (arrowhead) and the nucleus (NU) were observed (panel E). An epithelial cell from transgenic kidney demonstrated degeneration (arrowhead), absence of microvilli, and scarcity of cellular organella (panel F). Proteinaceous luminal fluid (FL) and degenerative cell debris (DB) were also visible. Original magnification was 7,930×.

genes *vif*, *vpr*, and *vpu* and the regulatory genes *tat*, *rev*, and *nef*. Three transgenic lines with identical renal disease have been characterized in detail [12]. Southern blots indicate that each line bears multiple proviral copies located at different integration sites. Only one of these lines, TgN(pNL43d14)F01Lom (referred to as Tg26), has been sustained in long-term breeding [13].

Quantitation of kidney DNA and protein content

Control and transgenic frozen kidneys were minced and processed in a solution of proteinase K and sodium dodecyl sulfate. Samples were deproteinized by successive phenol/chloroform/isoamyl alcohol extraction. DNA was recovered by ethanol precipitation, dried, and quantified [29] and protein concentration was measured in kidney homogenates [30].

Immunolocalization of bFGF in renal tissue

Immunohistochemistry was performed as previously described using polyclonal rat and human bFGF antibodies raised in rabbits and provided by Dr. Andrew Baird (Whittier Institute, La Jolla, CA) [18]. To facilitate penetration of the antibody into tissue, the sections were then washed in phosphate buffered saline (PBS) and treated with 1 mg/ml of hyaluronidase (type V: Sigma) buffered at pH 5.5 with 0.1 M sodium acetate containing 0.15 M NaCl, for 30 minutes at 37°C. Specific controls included: (1) substitution of normal rabbit sera for the primary antibody; (2) omitting the primary antisera; and (3) extracting sections with 2.0 M NaCl prior to the immunohistochemistry procedures.

Extraction of bFGF from renal tissue

bFGF was extracted from kidney samples as previously described [19]. Briefly, kidney samples were homogenized in 2% SDS/solubilization buffer (20 mM Tris-HCl, 1 mM EDTA, 1 mM EGTA pH 7.4, 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin A) centrifuged at 20,000 rpm, and incubated with heparin Sepharose 100 μl/ml for 18 hours. Samples were extracted with 2 M NaCl. For analysis of the composition of the extracts, samples were reduced with 50 mM dithiothreitol and electrophoresed through a 12.5% denaturing polyacrylamide gel. The electrophoresed proteins were transferred to nitrocellulose filters by electroblotting and were immunostained with a 1:1000 dilution of the bFGF rabbit polyclonal antibody.

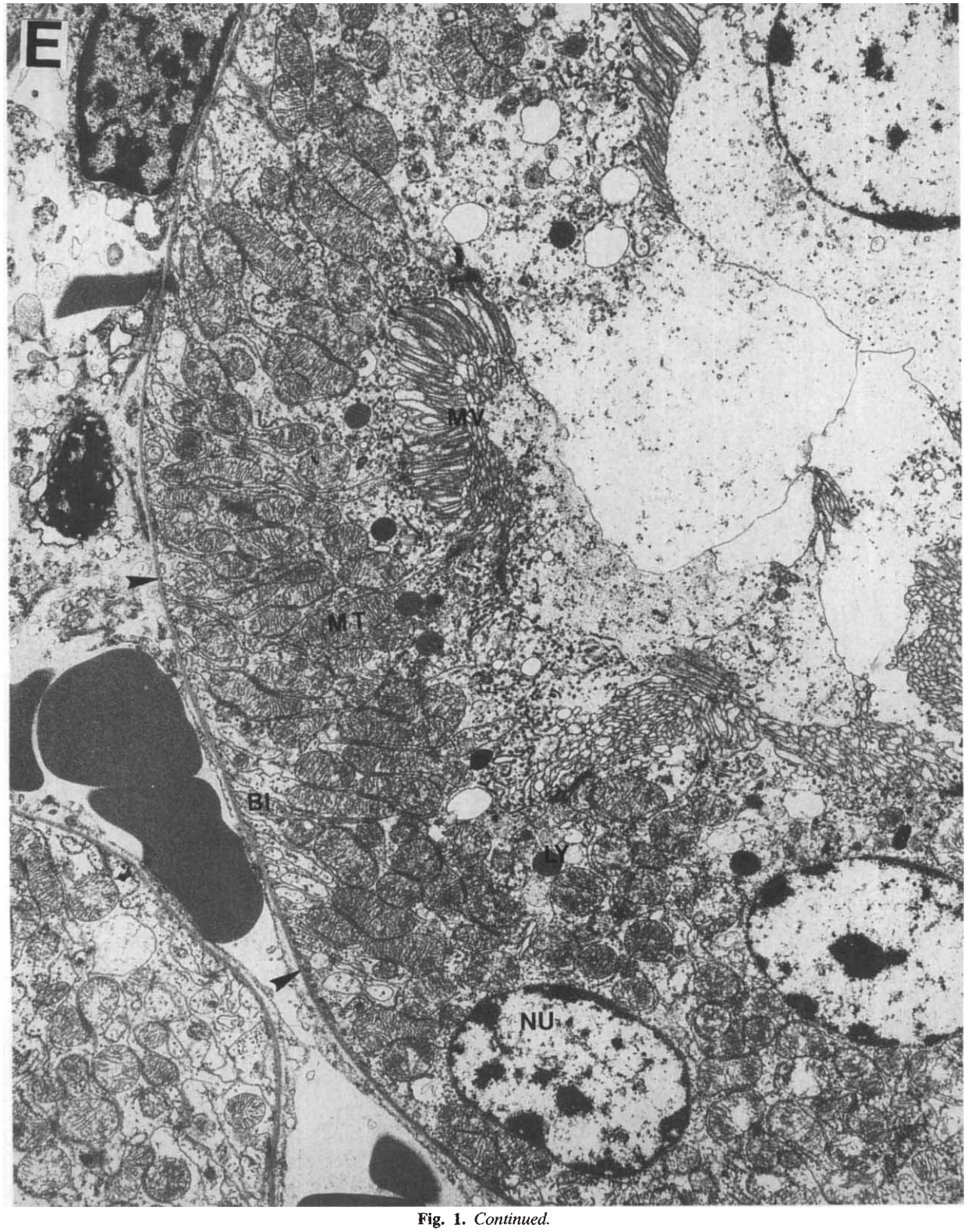


Fig. 1. *Continued.*

Preparation and analysis of RNA

Total cellular RNA was extracted from cultured cells [31]. PolyA+ mRNA was isolated from total RNA using a cellulose poly-dT column (Stratagene, La Jolla, California, USA) according to the manufacturer's instructions. For Northern analysis, polyA+ (2 μg) RNA was resolved on agarose/formaldehyde gels and

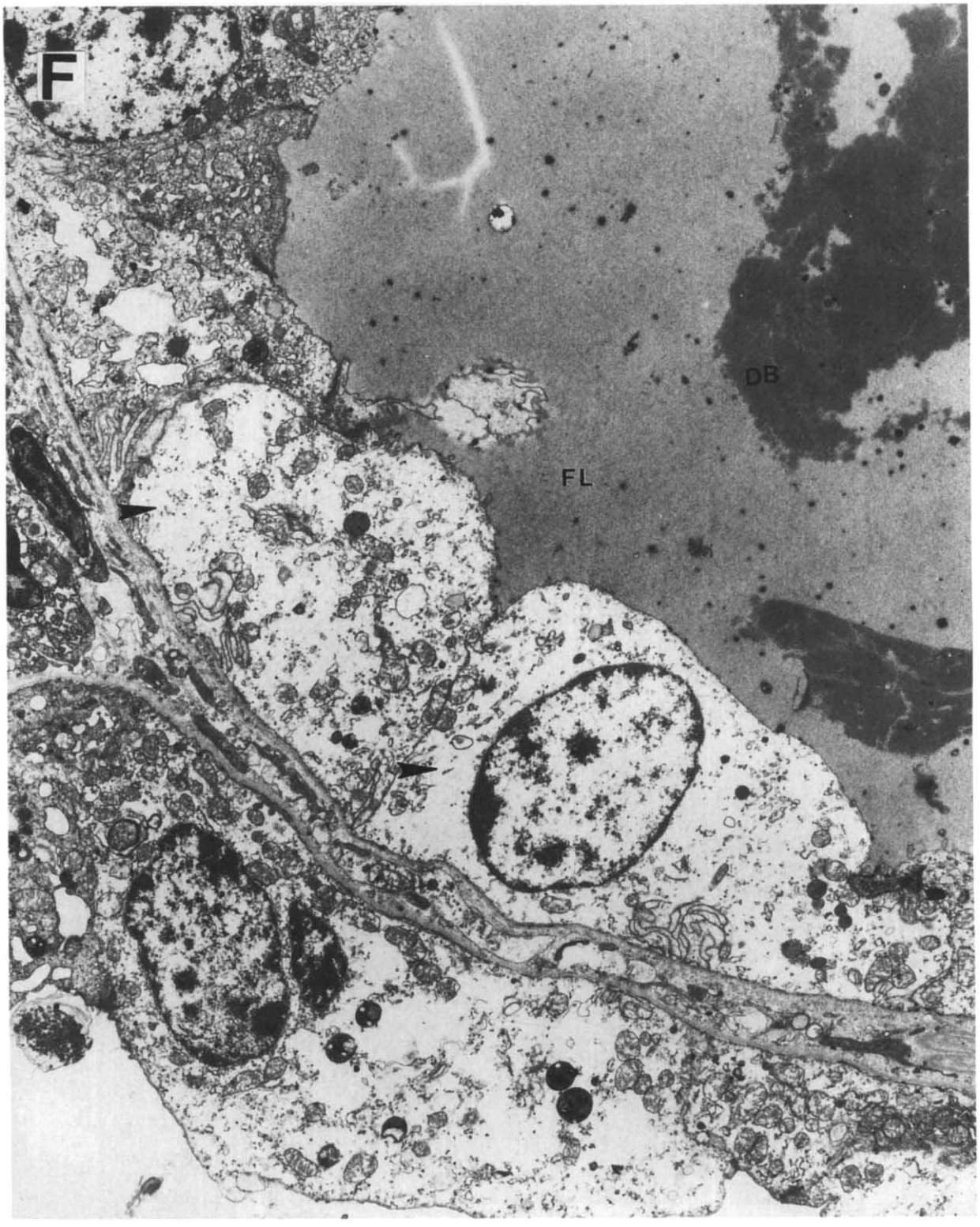
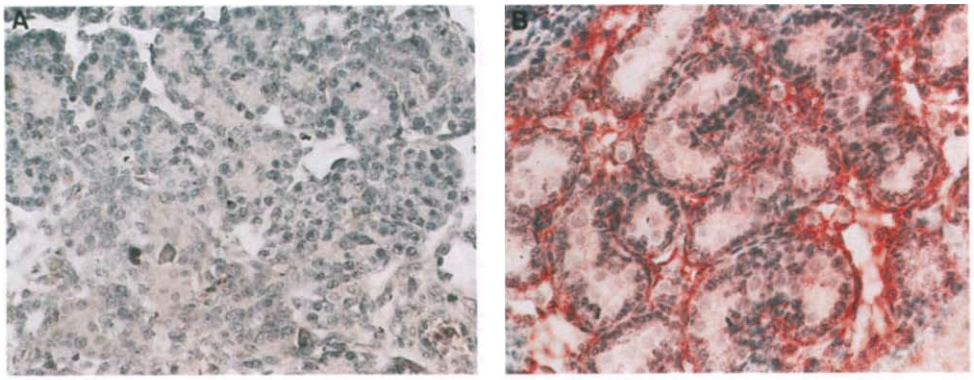


Fig. 1. *Continued.*

blotted to nylon membranes using positive pressure (Posiblot, Stratagene) [13]. RNA was cross linked by UV light, and membranes were then pre-incubated in a buffer composed of 50% formamide, 0.1% SDS, 5× SSPE, 100 μg/ml sheared salmon sperm DNA, and 2× Denhardt's solution for two hours at 42°C. [^{32}P]-labeled cDNA probes (10^7 cpm/filter) were hybridized for 18 hours at 42°C in the above buffer containing 10% dextran sulfate. After hybridization, the filters were washed twice in 6× SSPE,



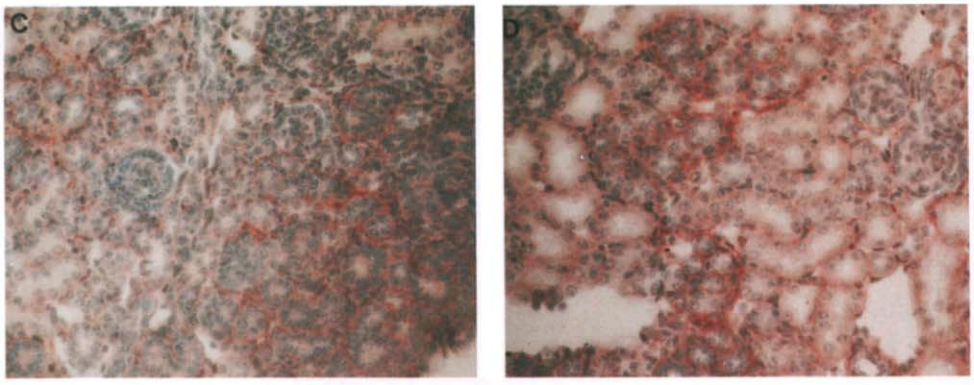
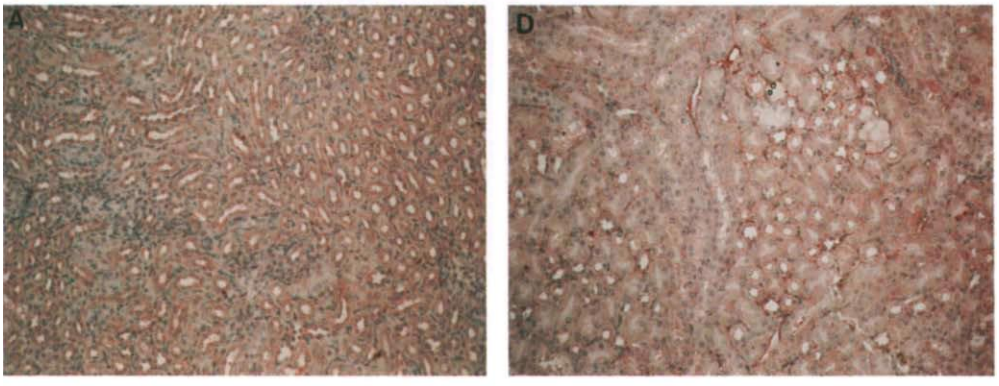
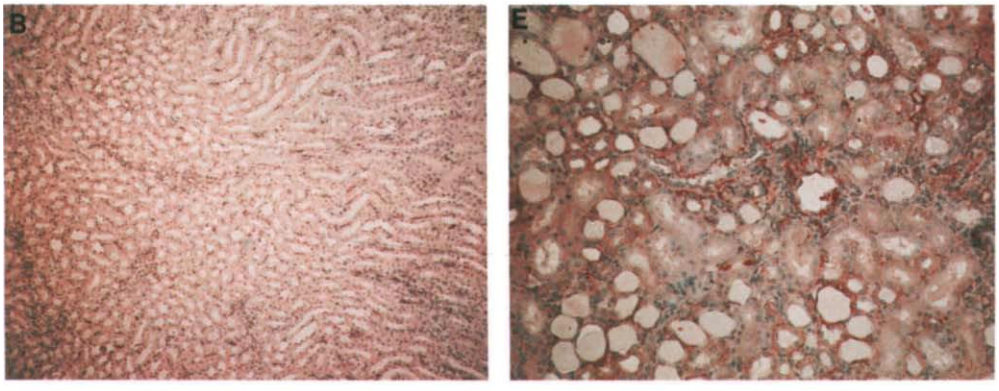


Fig. 2. *Immunolocalization of bFGF in control and transgenic fetal kidney.* Fetal kidney from transgenic mouse stained with normal rabbit serum as an antibody control (**A**). Fetal kidney from transgenic mouse demonstrated positive staining with an anti-bFGF antibody (2.5 μg/ml) that was evident in the extracellular matrix surrounding renal tubules (**B**). No significant differences in bFGF staining between nontransgenic control (**C**) and transgenic (**D**) fetal mouse kidney were observed. Original magnification in panels A and B was 640× and in panels C and D was 320×.





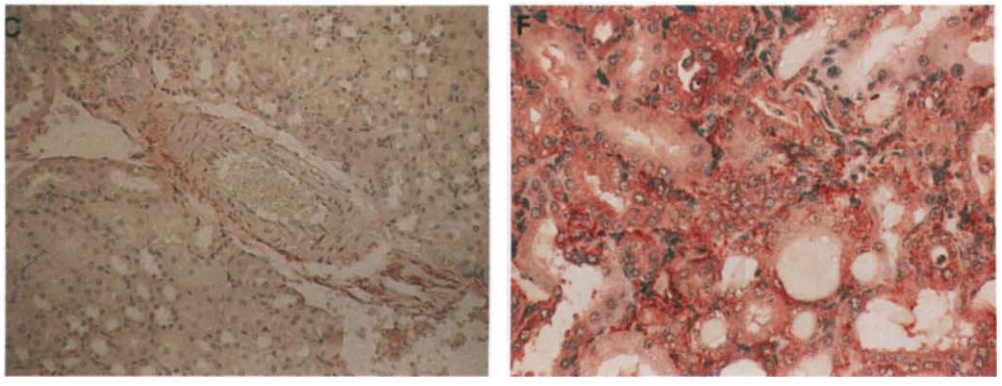


Fig. 3. *Immunolocalization of bFGF in adult kidney sections from control and transgenic mice.* bFGF staining was absent in renal medulla of 35-day-old (**A**) and 60-day-old (**B**) control mice. Immunoreactive bFGF was localized to major renal arteries in control mice (**C**). Basic FGF staining was detected in the extracellular matrix surrounding renal tubules and microcysts in 35-day-old (**D**) and 60-day-old transgenic mice (**E** and **F**). Original magnification in panels A, B, D, and E was 160×, in panel C 320×, and in panel F 640×.

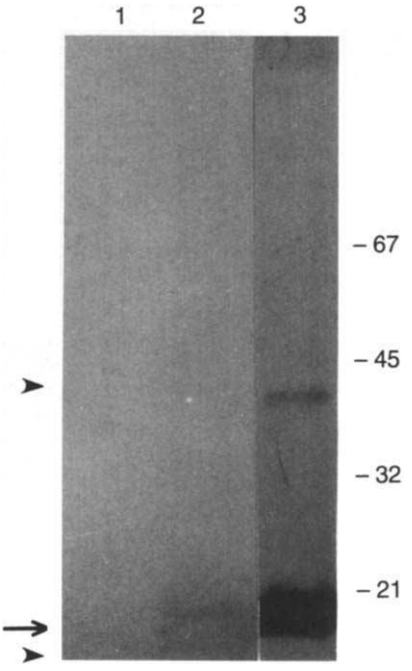


Fig. 4. *bFGF Western blot analysis of normal and transgenic kidney extracts.* Basic FGF was extracted from normal (lane 1) and transgenic (lane 2) kidney homogenates, and concentrated by adsorption on to heparin-sepharose as described in **Methods**. Each sample containing 60 μg of protein was reduced with 50 mM dithiothreitol and electrophoresed through a 12.5% denaturing polyacrylamide gel. The electrophoresed proteins were transferred to nitrocellulose filters by electroblotting and were immunostained with a 1/1000 dilution of bFGF rabbit polyclonal antibody. Recombinant bFGF (50 ng, Biosource International) is provided as a positive control (lane 3). bFGF was not detected in kidney extracts from normal control mice that were 60 days old (lane 1). In transgenic mouse kidneys, however, a bFGF immunoreactive band (large arrow) was detected co-migrating with control human recombinant bFGF (lanes 2 and 3, respectively). Two additional weakly immunoreactive bands could be detected above and below the major band (arrowheads), which also co-migrated with equivalent bands in the recombinant bFGF lane. The larger molecular weight bands in lanes 2 and 3 are consistent with previous reports that bFGF can exist as a dimer under SDS and reducing conditions. The identity of the smaller molecular weight band is unknown.

0.1% SDS for 15 minutes at room temperature, twice in 1× SSPF, 0.1% SDS for 15 minutes at room temperature, and twice in 0.1× SSPE, 0.1% SDS for 15 minutes at 45°C. The probe specific for all HIV-1 mRNAs was a *nef* cDNA 1.1kb *Bam*HI fragment from HIV-1_{HXB-2} (NIAID Research and Reference Reagent Program). This probe overlaps the proviral 3′ LTR and is, therefore, expected to hybridize will all proviral mRNA [32]. The bFGF probe was a 0.8 kb *Eco*RI fragment from a cDNA clone [33].

bFGF in vitro autoradiography

Mouse kidneys were dissected and immediately frozen in isopentane on dry ice. Frozen mouse kidney sections (16 μm) were cut in a cryostat at −14°C, mounted onto cold gelatin-coated glass microscope slides, and stored under vacuum at 4°C for 12 hours. *In vitro* autoradiography was performed as described previously [34, 35]. Briefly, sections were preincubated for 15 minutes in binding buffer (DMEM, 20 mM HEPES at pH 7.4, and 0.15% gelatin) followed by incubation in the same buffer containing 2, 5, or 10 ng/ml [^{125}I]-bFGF (Amersham Corp., Arlington Heights, Illinois, USA) at 4°C for two hours. Nonspecific binding was determined by incubating sections with 5 μM unlabeled bFGF plus 30 μg/ml heparin (Fisher Scientific, Fair Lawn, New Jersey, USA) or 300 μg/ml heparin alone. Heparin was used to displace binding because addition of unlabeled bFGF alone did not completely displace labeled bFGF [36]. After incubation, the slides were rinsed consecutively four times for one minute each in fresh binding buffer (4°C). Binding to low affinity sites was detected after washing tissue sections with 2 M NaCl, 20 mM HEPES, pH 7.4 for one minute. Finally, kidney sections were washed in ice-cold water and dried under cold forced air. Film exposure time ranged from one to four days at room temperature. Films were developed with D19 Kodak developer for five minutes at 4°C. In each experiment, equal amounts of iodinated growth factor or heparin were added to control or transgenic kidney sections, incubated under the same conditions, and autoradiographs were exposed for equal lengths of time. Differences in binding were determined by optical density of the autoradiograms by computerized microdensitometry in specific kidney areas of 0.22 mm^2 as described before [34]. Protein content of the specific areas used for receptor quantification in kidney was determined by a densitometric procedure described previously [34].

Cell culture and proliferation assays

Primary cultures of RTECs were obtained from control and transgenic mice using standard methods [37]. Renal medullary tissue was minced and passed through serial sieves. Fractions containing tubular fragments were washed with Hanks balanced salt solution, and digested with 0.1% collagenase (Type IV-S, Sigma, St. Louis, Missouri, USA) for 30 minutes at 37°C. The released cells were plated onto collagen-coated plastic dishes in Dulbecco's modified Eagle's medium (Biofluids, Rockville, Maryland, USA) supplemented with insulin (5 μg/ml), transferrin (5 μg/ml), selenium (5 ng/ml), hydrocortisone (36 ng/ml), triiodothyronine (4 pg/ml), and epidermal growth factor (10 ng/ml).

At confluence, these cells exhibited characteristic epithelial morphology, growing in a monolayer with a cobble-stone appearance. Confluent RTECs stained positively for keratin and formed "domes." They lacked extensive actin intermediate filaments (characteristic of fibroblasts) or Factor VIII-related antigen (characteristic of endothelial cells). Transgenic renal fibroblasts were isolated by clonal dilution from cells from the renal medulla [38]. NIH 3T3 cells were grown in DMEM containing 10% calf serum (Gibco, Grand Island, New York, USA). Bovine aortic endothelial cells or human umbilical endothelial cells (provided by Dr. D. Grant NIDR/NIH) were grown in tissue culture as previously described [36, 39, 40].

Heparin-sepharose (Pharmacia Diagnostics, Uppsala, Sweden) column chromatography (column size 1 ml) was performed as previously described [18–20]. Briefly, 3 ml of concentrated conditioned medium (5×) was applied to the column and washed with 20 mM Tris-HCl (pH 7.4). The column was eluted with a linear gradient of 0.2 to 2.0 M NaCl in Tris-HCl (pH 7.4). Each fraction was concentrated fivefold by Centricon 10 microconcentrators (Amicon, Beverly, Massachusetts, USA) with a molecular weight cut off of 10 kDa. Concentrated samples (20 μl) were assayed for mitogenic activity by [^{3}H] thymidine incorporation of target cells as described previously [41]. Briefly, cells were made quiescent for 24 to 48 hours in the absence of serum or other growth factors and pulsed with [^{3}H]thymidine for 6 to 24 hours.

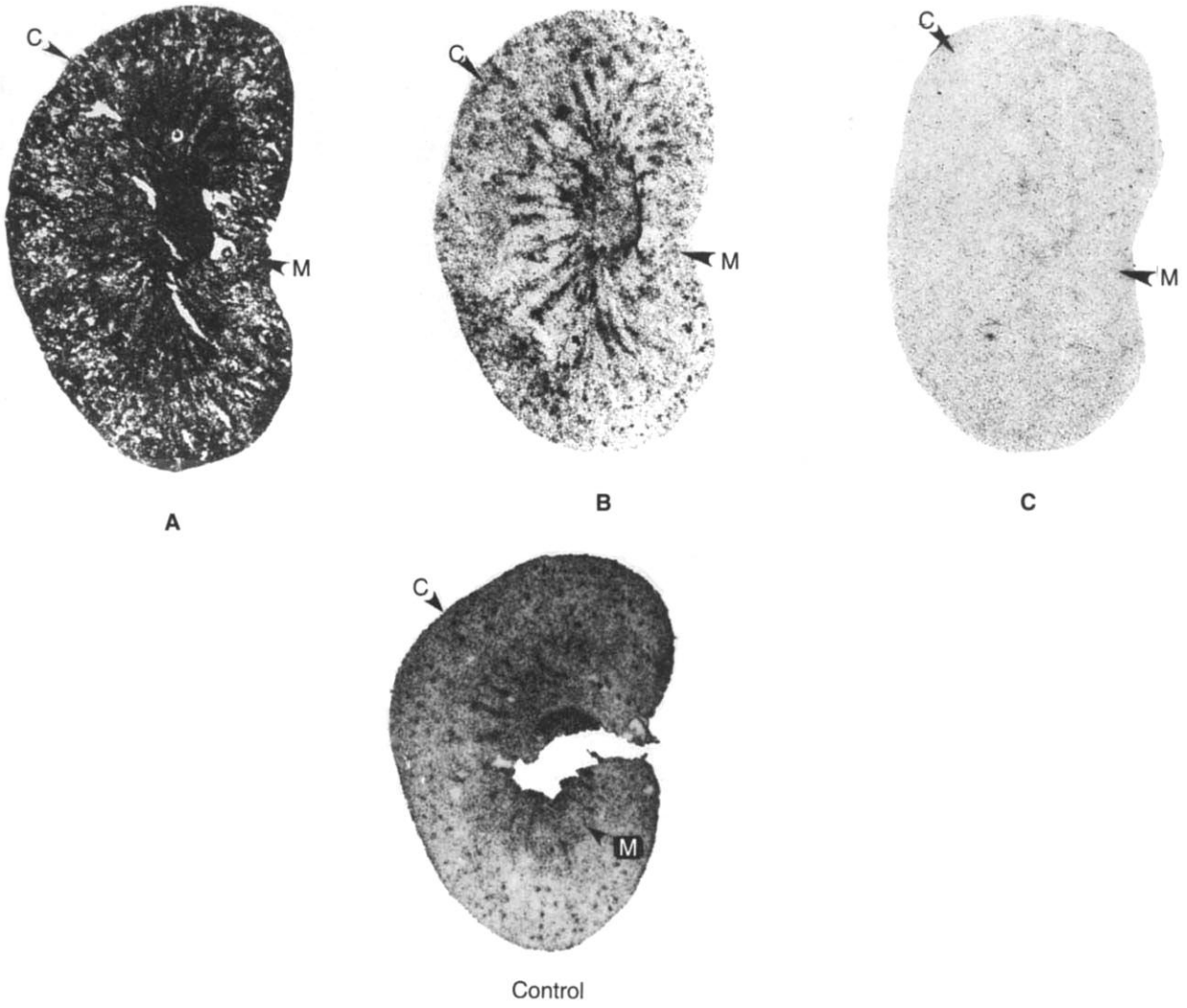


Fig. 5. In vitro *autoradiography for bFGF binding sites using [^{125}I]-bFGF.* bFGF binding sites in control kidney sections from 60-day-old mice localized to glomeruli in the renal cortex (C) and vasa recta in the renal medulla (M). In transgenic kidney, the distribution of bFGF binding sites was similar but increased, particularly in the renal interstitium (**A**). In transgenic kidney, higher affinity binding sites, detected in the presence of heparin (30 μg/ml), were found predominantly in vasa recta and glomeruli (**B**). bFGF binding could be displaced completely at a higher heparin concentration (300 μg/ml) suggesting that all renal bFGF binding sites were sensitive to heparin displacement (**C**). These findings were identical in 7 control and transgenic mice.

After that period, the cells were precipitated with 5% TCA and solubilized with SDS. Aliquots were counted and results were expressed in counts per minute (cpm).

Growth of renal tubular epithelial cells on matrigel

For regeneration experiments, RTECs were cultured on matrigel (provided by Dr. Hynda Kleinman), a reconstituted basement membrane extracted from EHS mouse sarcoma tumor as previously described [40]. Plastic culture wells (16 mm) were coated with matrigel, 250 μl of a 10 mg/ml solution and the matrigel was allowed to solidify at 37°C for one hour. RTECs (55,000 cells/well) from control and transgenic mice were cultured in DMEM without serum, heparin, or growth factors. Additionally, other nontransgenic RTECs were pretreated with bFGF (20 ng/ml for 24 hr) prior to plating on matrigel. Twenty-four hours after culturing RTECs on matrigel, the number of formed tubules was counted in 5 microscopic fields (2×) of 10 different wells. In some cultures, cells were fixed in glutaraldehyde and analyzed by transmission electron microscopy.

Statistical analysis

Results are expressed as the mean ± SD of values obtained in triplicate from at least three different experiments. Difference between groups was compared by Student's *t*-test. *P* values less than 0.05 were considered significant. When more than two means were compared, significance was determined by one way analysis of variance followed by multiple comparisons using the Student-Neuman-Keul's test.

Results

Renal disease in HIV transgenic mice

Tubulointerstitial disease is the most striking feature of the transgenic kidney with varying degrees of tubular distortion and

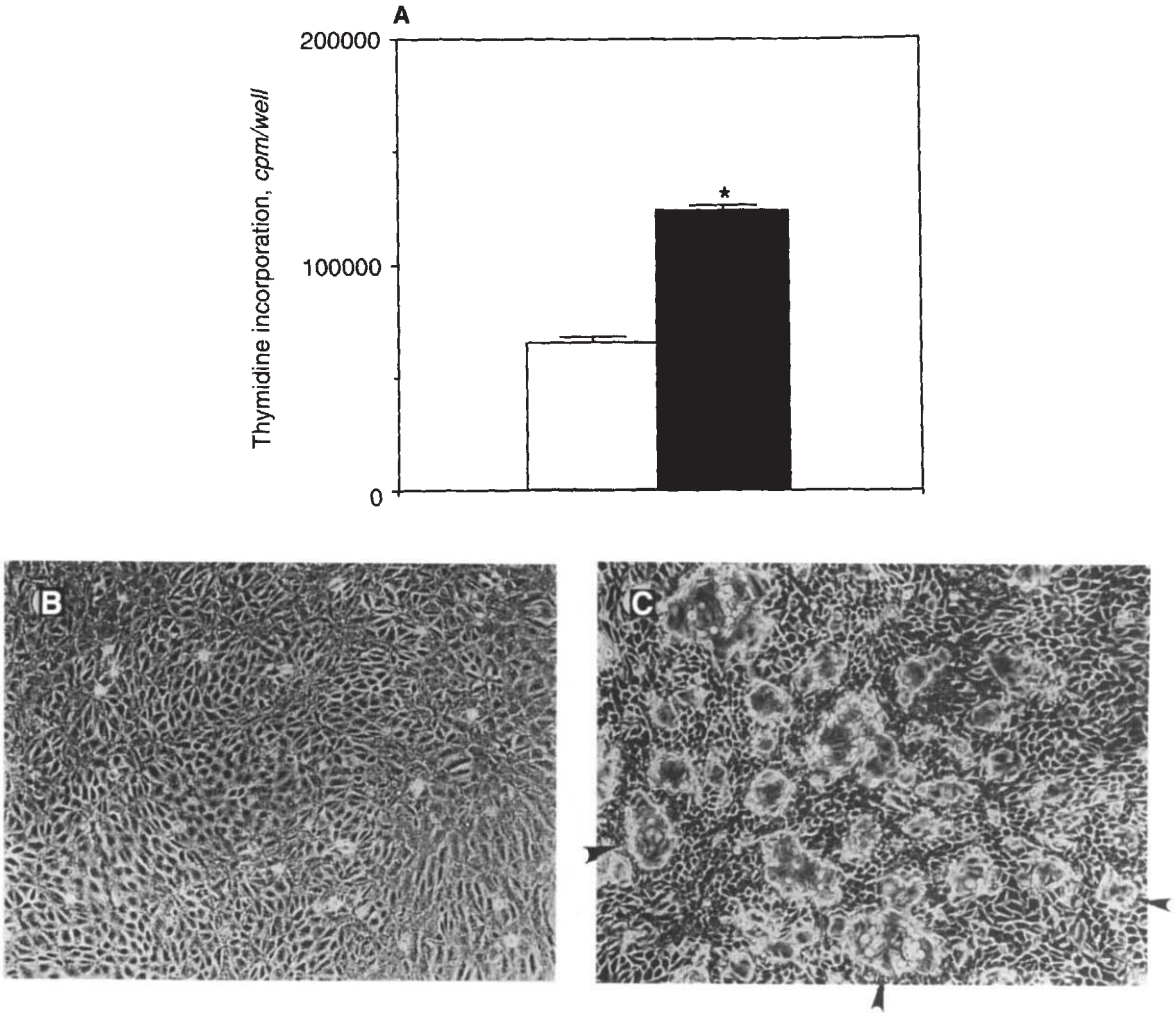


Fig. 6. *Spontaneous proliferation of renal tubular epithelial cells (RTECs) from control and transgenic mice.* DNA synthesis (thymidine incorporation) of primary cultures of RTECs is shown in panel **A**. Equal number of control and transgenic RTECs were plated as described in methods. After 6 days in culture, cells were incubated with 1 μCi [^{3}H]-thymidine for 24 hours. Spontaneous proliferation of transgenic RTECs was significantly greater than control cells. Similar results were obtained in four separate experiments with cells isolated from different animals. The asterisk indicates $P < 0.001$. Phase contrast light micrographs of confluent RTECs isolated from 60-day-old nontransgenic mice (**B**) and transgenic mice with renal disease (**C**) are shown. Increased dome formation, a characteristic feature of RTECs, is indicated by the arrowheads.

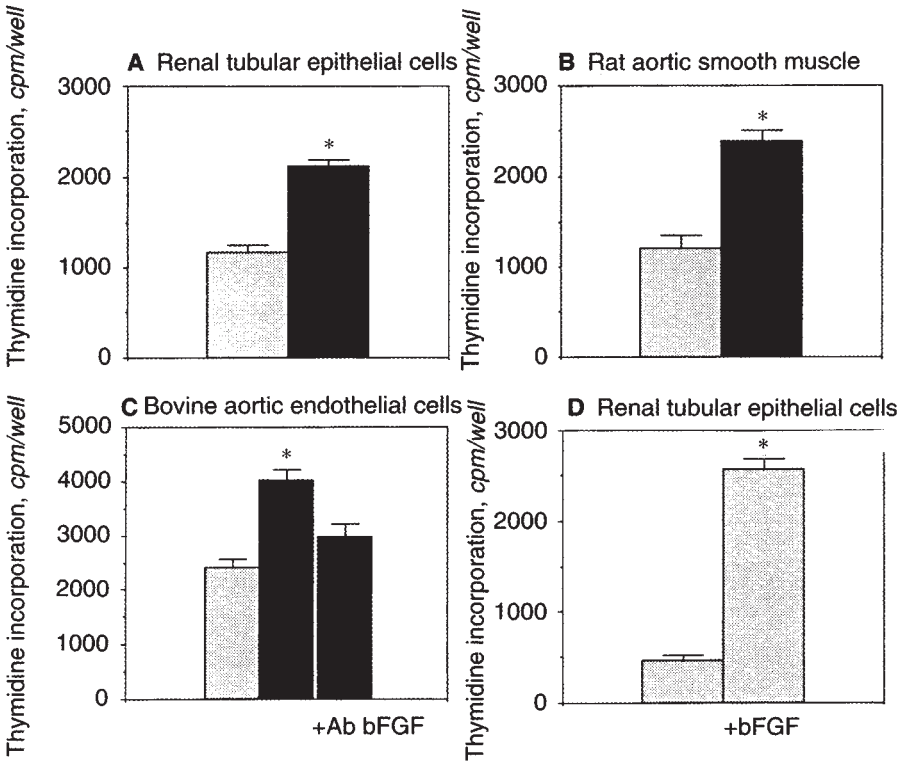
dilatation as well as simultaneous evidence of cell injury, regeneration, and proliferation. Proliferation of renal tissue was evident even by gross pathological observation at 60 days of age; kidneys were enlarged with a cobblestone appearance of the surface (Fig. 1A). Tubulointerstitial distortion was dramatic and characterized by microcyst formation (Fig. 1 B, C, D). In addition to tubular degeneration, there was evidence of coexistent regeneration with a greater number of nuclei and mitotic figures in renal interstitium. By 60 days of age, kidney weights were significantly increased in transgenic kidneys when compared to littermate controls (220 ± 28 vs. 151 ± 21 respectively; $P < 0.05$). Both protein and DNA content of transgenic kidneys were significantly increased by an average of 24% and 25%, respectively, when compared to control. Immunostaining for proliferating cell nuclear antigen (PCNA) showed a greatly increased number of proliferative cells including RTECs, spindle-shaped interstitial cells, and in some sections, mononuclear cells in perivascular aggregates. PCNA positive cells and mitotic figures were found in areas of microcystic dilation and bFGF localization (data not shown).

Renal tubular epithelial cell injury

To determine the ultrastructural characteristics of RTEC, we examined the renal epithelium by transmission electron microscopy. There was striking focality of the injury process. As shown in Figure 1 E and F, RTECs revealed evidence of degenerative changes with decreased number of mitochondria, mitochondrial swelling with loss of cristae, and tubular atrophy with loss of microvilli. These cells were generally found adjacent to normal cells.

Distribution of bFGF in renal tissues

To explore the distribution of bFGF in transgenic kidneys, we performed immunohistochemistry using transgenic and control



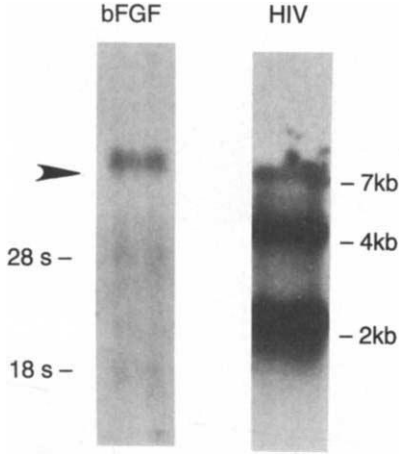


Fig. 7. *Characterization of bFGF-like activity in conditioned medium from transgenic renal tubular epithelial cells (RTECs) after heparin-sepharose purification.* Several cell types were used to characterized the proliferative effects of eluate fractions. All mitogenic effects were tested in the absence of heparin. The mitogenic effect of a concentrated fraction (5×) eluted from a heparin-Sepharose column with a 1.5 to 2.0 M NaCl gradient is shown in panel A. Subconfluent transgenic RTECs were used as target cells. A similar fraction eluted from the heparin-Sepharose column was used to assay proliferative response of subconfluent rat aortic smooth muscle cells (**B**) and subconfluent bovine aortic endothelial cells (**C**). A 1:20 dilution of a polyclonal rabbit antibody to bFGF neutralized the proliferative effects of this fraction. The mitogenic effect of bFGF (20 ng/ml, Biosource International) on transgenic RTECs in subconfluent primary culture is shown in panel **D**. No additional growth factors or heparin were added to RTEC cultures. The asterisk indicates significance at $P < 0.05$. The expression of bFGF and the HIV-1 transgene by RTECs isolated from 60-day-old mouse kidney and analyzed by Northern blot is shown in panel **E**. A 6.8 kb transcript (arrowhead) corresponding to bFGF was detected in transgenic RTECs. RTECs also expressed HIV-1 mRNA of the three expected size classes, 2kb, 4kb, and 7 kb that correspond to multiply spliced, singly-spliced, and full-length transgene mRNAs, respectively.

kidneys at various ages. During renal development, bFGF could be detected easily in the interstitium surrounding renal tubules (Fig. 2 B, C, D). No significant differences were present between control and transgenic fetal kidneys (Fig. 2 C and D). At 35 days of age, however, there were marked differences in the distribution of bFGF (Fig. 3). bFGF was not detected in the interstitium of 35 day old control animals (Fig. 3A). With the development of interstitial renal disease in 35-day-old transgenic mice, bFGF could be detected surrounding tubules (Fig. 3D). At 60 days of age, bFGF was significantly increased in extracellular matrix surrounding areas of microcystic dilatation and in areas of interstitial proliferation in transgenic mice (Fig. 3 E, F). In age-matched control mice, no bFGF was detected in renal interstitium (Fig. 3B). Normal localization of bFGF in medullary renal arteries was detected in sections from adult nontransgenic animals (Fig. 3C).

Quantitation of renal bFGF

To determine whether bFGF was increased in renal extracts of diseased transgenic kidneys *in vivo*, we performed Western blot analysis with transgenic and control kidney extracts (Fig. 4). Three bFGF immunoreactive bands were detected in protein extracts from transgenic mouse kidney that comigrated with recombinant

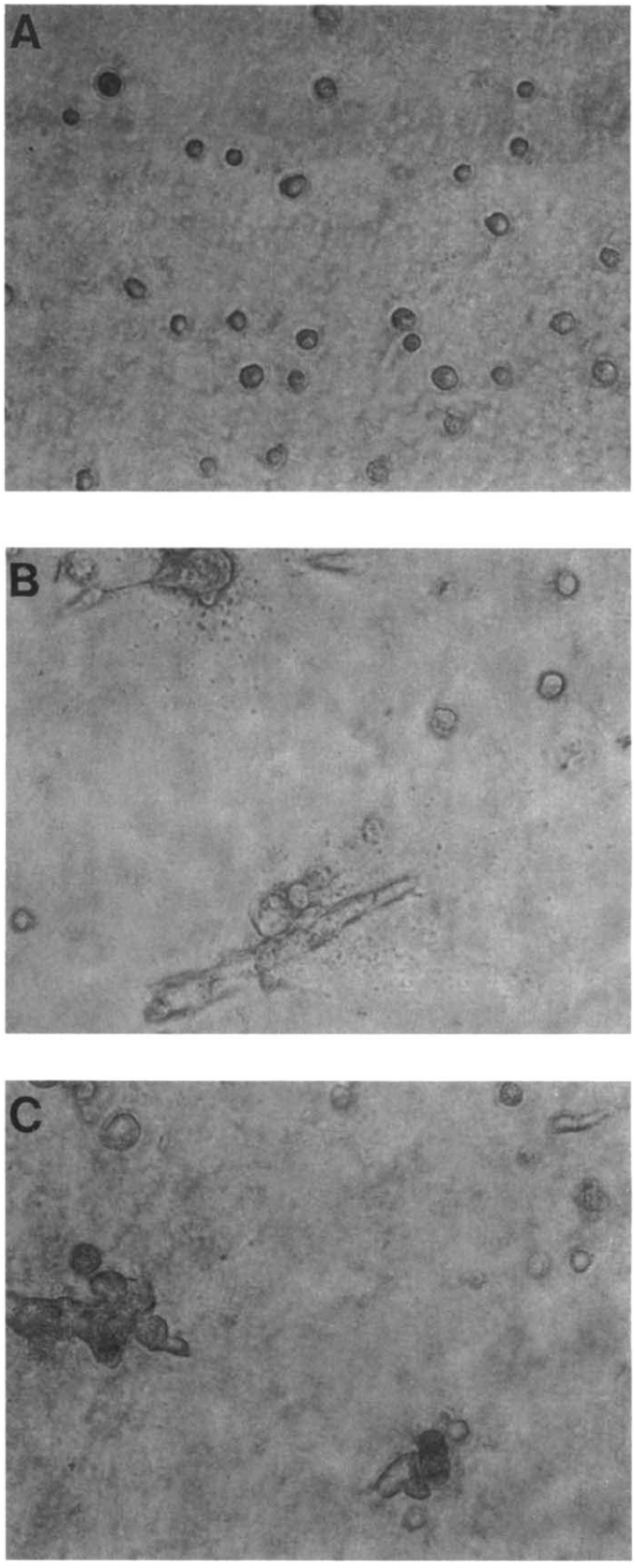


Fig. 8. *Tubule regeneration by renal tubular epithelial cells (RTECs) in culture.* RTECs isolated from control mice remained round in shape without evidence of spreading after 24 hours of culture on a matrigel substrate (**A**). In contrast, transgenic RTECs (**B**) or RTECs isolated from nontransgenic control mice that were pre-treated with bFGF (**C**) formed tubular structures on matrigel. Cells were photographed using Hoffman modulation contrast on a Nikon inverted light microscope (magnification 560×). Transmission electron microscopy of a tubular structure formed by transgenic RTECs is shown in panel **D**. The tubule formed in vitro had a patent lumen (L) with cellular debris (DB), a basement membrane (arrowheads), and tight junctions (arrows) (original magnification 7,930×).

bFGF; bFGF was not detected by Western blot in control kidneys. By Northern analysis, expression of bFGF mRNA was very low and no difference in steady state mRNA could be detected (data not shown).

bFGF low affinity binding sites in renal tissue

Since bFGF binds to components of the extracellular matrix, particularly heparan sulfate proteoglycans, we examined whether the increased accumulation of bFGF was due to its binding to these low affinity sites in renal tissue. To determine the renal distribution of bFGF low affinity binding, we performed experiments on frozen renal sections using [^{125}I]-bFGF. Total bFGF binding in renal cortex and medulla was significantly increased in the transgenic kidneys (Fig. 5A) when compared to controls (Fig. 5 control). Binding to these sites could not be completely displaced by even a 100-fold excess of unlabeled bFGF, suggesting a very large reservoir of bFGF low affinity binding sites (data not shown). When sections were incubated in the presence of labeled bFGF and low concentrations of heparin (30 μg/ml heparin), bFGF binding to low affinity receptors was significantly reduced (Fig. 5B) when compared to sections incubated without heparin (Fig. 5A). Higher affinity binding sites were found overlying renal glomeruli and along the vasa recta in renal medulla (Fig. 5B). These higher affinity binding sites were significantly increased in the renal cortex and medulla of transgenic kidneys (control cortex 97 ± 9 vs. transgenic cortex 165 ± 11 OD units; $P < 0.05$ and control medulla 107 ± 12 vs. transgenic medulla 215 ± 23 OD units; $P < 0.05$). Binding sites for bFGF in renal cortex, particularly glomeruli, and along the vasa recta in renal medulla correlated with proteoglycan distribution in these areas by Alcian Blue staining at pH 1.0 (data not shown) and by immunohistochemistry for heparan sulfate proteoglycan [13]. bFGF binding could be displaced completely at a higher heparin concentration (300 μg/ml) suggesting that all renal bFGF binding sites were sensitive to heparin displacement (Fig. 5C). Pretreatment of kidney sections with heparitinase (Seikagaku America, Rockville, Maryland, USA) at a concentration of 0.01 U/ml for one hour at 37°C completely abolished binding of bFGF to renal glomeruli and along the vasa recta in renal medulla.

Renal tubular epithelial cell proliferation

To explore whether bFGF present on the cell surface of the RTEC could affect epithelial growth, RTECs were isolated from control and transgenic kidneys with interstitial disease and growth characteristics were determined *in vitro* (Fig. 6). Cells expressing the transgene *in vitro*, demonstrated spontaneous proliferation at a more rapid rate than control cells. Figure 6A shows results of thymidine incorporation (control: 72,000 ± 2,340 vs. transgenic: 127,000 ± 5,234 cpm/well $P < 0.05$) and Figures 6 B and C cell number (control: 79,345 ± 7,562 cm^2 cells/well vs. transgenic: 153,230 ± 12,245 cells/cm^2; $P < 0.05$).

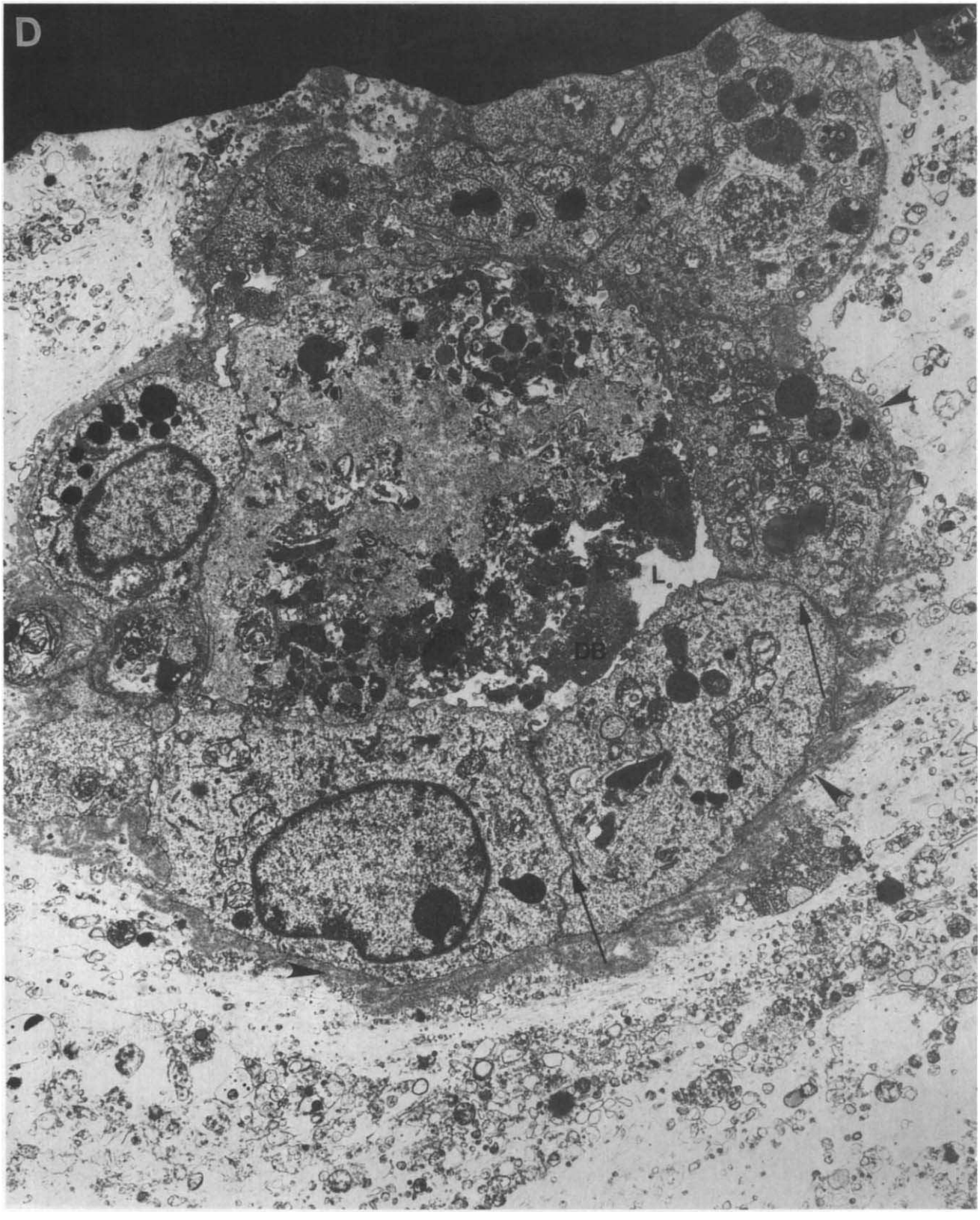


Fig. 8. *Continued.*

Identification of bFGF in conditioned medium

To identify whether biologically active bFGF is present in conditioned medium from normal and transgenic diseased kidneys, primary RTEC isolates were cultured *in vitro*. Conditioned medium from control and transgenic cells was collected and used to stimulate proliferation of several cell targets. Only concentrated (5×) conditioned medium from transgenic RTECs stimulated the growth of renal epithelial, renal fibroblasts, rat aortic

smooth muscle cells, and bovine and human endothelial cells; conditioned medium from control cells had no significant proliferative effects on endothelial cells. Heparin sepharose column chromatography was performed to characterize further the bFGF activity (Fig. 7). Fractions eluted from the heparin sepharose column with 1.5 to 2.0 M NaCl were able to stimulate proliferation of all cell types in the absence of heparin (Fig. 7 A, B, C). Antibody directed against bFGF inhibited bovine aortic endothelial cell proliferation in response to conditioned medium (Fig. 7C). Furthermore, bFGF in the absence of other growth factors, increased thymidine incorporation of primary renal epithelial target cells approximately fivefold (Fig. 7D) as well as the other cell lines tested.

bFGF and HIV transgene expression

To ensure that bFGF was expressed by transgenic RTECs *in vitro*, we performed Northern analysis of transgenic renal cells in culture following selection for poly A+ RNA (Fig. 7E). RTECs in culture expressed the 6.8 kb transcript of bFGF as well as the proviral mRNA of the three size classes expected, 2kb, 4kb and 7 kb.

bFGF and renal tubular epithelial cell regeneration

Although we had evidence that bFGF could induce epithelial cell proliferation, it remained unclear whether bFGF could stimulate tubular regeneration. To explore this issue, we cultured RTECs on matrigel, an artificial basement membrane derived from EHS tumor. Matrigel has previously been shown to sustain neonatal mouse RTECs in a differentiated phenotype [42]. Transgenic, nontransgenic, and nontransgenic RTECs pretreated for 24 hours with bFGF were cultured on matrigel. As shown in Figure 8, RTECs isolated from transgenic mice (Fig. 8B) formed tubule-like structures on matrigel within 24 hours of plating, in contrast to RTECs isolated from normal mice (Fig. 8A). As shown in Figure 8D, transmission electron microscopy revealed that these structures were developing tubules with tight junctions, surrounding basement membrane, and patent lumens. Control cells pretreated with bFGF and plated on matrigel (Fig. 8C) formed tubules similar to those observed with transgenic RTECs (Fig. 8B). These results suggest that bFGF participates in both RTEC proliferation and tubule regeneration in transgenic kidneys.

Discussion

Renal disease is increasingly recognized as a complication of HIV-1 infection, particularly in African Americans [10]. Although many of the sequelae of HIV-1 infection are related to immunosuppression, recent evidence supports a direct role for HIV-1 proteins in the pathogenesis of HIVAN [5, 12, 13]. In humans, HIVAN presents as nephrotic syndrome with no apparent correlation with $CD4^+$ T cell number. Characteristically, clinical progression to end-stage renal disease is rapid. Histologically, two essential features appear to dominate the renal pathology: cellular proliferation and accumulation of extracellular matrix. Microcystic distortion of the renal interstitium is often prominent and infiltrating inflammatory cells are common. Despite these well-established pathological features, the mechanisms responsible for these changes are unknown.

Recently, we have developed a transgenic mouse model of HIVAN. In this model, we have introduced as a transgene a deletion mutant of the infectious proviral clone pNL4-3. This mutant has a deletion of the *gag-pol* genes but encodes envelope and the regulatory and accessory genes under the control of the HIV-1 5′ LTR. Since three founders generated offspring with identical manifestations of renal disease but with different integration sites by restriction length polymorphism [12], integration interruption of a critical gene is not the cause of this disease. Instead, expression of HIV-1 proteins is sufficient to generate the pathological features of HIVAN in this mouse model in the absence of other risk factors [2–8] and in the absence of viral replication [12, 13]. In the present study, we have found that the proliferative tubulointerstitial changes characteristic of the microcystic changes of HIVAN are associated with increased expression of low affinity binding sites for bFGF in renal interstitium. This increase in low affinity binding sites facilitates the accumulation of bFGF in areas of cellular injury and may be critical for inducing RTEC proliferation.

RTEC injury in transgenic kidney occurred in cells that expressed both the HIV-I transgene as well as bFGF. Unlike most other polypeptide growth factors, bFGF lacks a conventional signal sequence for secretion and, as a result, the mechanisms responsible for the release of bFGF remain unclear. With cells that are chronically injured, however, bFGF is efficiently released [43]. Furthermore, tissue injury potentiates the mitogenic effects of bFGF [22]. These regenerative processes may be particularly relevant in HIVAN. In the present study, accumulation of bFGF appeared to be associated with areas of the renal interstitium that contained both cell injury and proliferation. We have not excluded the possibility, however, that other growth factors [44] or HIV-encoded proteins may have had an additional role in proliferation as well.

In the present study we found significant alterations in the distribution and affinity of bFGF binding sites in transgenic kidney. Previous studies have suggested that the kidney is an important site for the uptake of exogenously administered bFGF [45], although the renal distribution of these binding sites has remained unclear. We have now demonstrated that bFGF binding sites were localized to renal glomeruli and vasa recta in the rena' medulla. The finding in glomeruli is consistent with other studies that have demonstrated that glomerular visceral epithelial cells synthesize both bFGF [23] and HSPG [46]. Thus, this epithelial cell type possesses a mechanism for the modulation of the local biological activity of bFGF. Lower affinity binding sites were found in the renal interstitium of transgenic kidneys in the same distribution as heparan sulfate proteoglycans. Precedence for HSPG as a bFGF receptor certainly exists in other biological systems [47]. Furthermore, heparan sulfate proteoglycans, acting as bFGF low-affinity receptors [48, 49], may affect the interaction of bFGF with its high affinity receptor and, as a result, regulate biological activity. Extrapolating from the findings of our current study, the large number of low affinity bindings sites in kidney may be critical for the clearance of bFGF from the circulation particularly in neoplastic diseases characterized by increased circulating levels of bFGF [27, 28, 39]. Thus, it is possible that in addition to the local release of bFGF, extrarenal sources may also contribute to the deposition of bFGF in kidney.

Proteoglycans have been increasingly recognized as modulators of renal cell growth [49, 50], and our findings again support an important role for these extracellular matrix proteins in the pathogenesis of proliferative tubulointerstitial changes resulting from HIV-1 infection. TGF-β may play an important role in this

process as well since TGF-β modulates the production of renal proteoglycans which bind bFGF [51]. Since TGF-β inhibits the growth of RTECs *in vitro*, it is unlikely that a direct effect of TGF-β alone is responsible for the tubulointerstitial proliferative changes characteristic of HIVAN. By stimulating the proliferation of renal fibroblasts which produce extracellular matrix proteins, however, bFGF may affect both the proliferative and fibrotic processes.

In summary, HIVAN appears to be the result of two distinct pathological processes, interstitial proliferation and the accumulation of extracellular matrix proteins. In the HIV-1 transgenic mouse model, an interaction between bFGF and its low affinity receptors appears to link these two processes. Thus, bFGF appears to play a critical role in the renal proliferation and microcystic distortion characteristic of HIV-induced renal disease and may have a similar role in other renal diseases as well.

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References

1. Schaffer RM, Schwartz GE, Becker JA, Rao TK, Shih YH: Renal ultrasound in acquired immune deficiency syndrome. *Radiology* 153:511–513, 1984
2. Rao TK: Clinical features of human immunodeficiency virus associated nephropathy. *Kidney Int* 35:S13–S18, 1991
3. Bourgoignie JJ, Pardo V: The nephropathology in human immunodeficiency virus infection. *Kidney Int* 35:S19–S23, 1991
4. Strauss J, Abitbol C, Zilleruelo G, Scott G, Paredes A, Malaga S, Montane B, Mitchell C, Parks W, Pardo V: Renal disease in children with the acquired immunodeficiency syndrome. *N Engl J Med* 321:625–630, 1989
5. Cohen AH, Sun NC, Shapshak P, Imagawa DT: Demonstration of human immunodeficiency virus in renal epithelium in HIV-associated nephropathy. *Mod Pathol* 2:125–128, 1989
6. Bourgoignie JJ, Meneses R, Ortiz C, Jaffe D, Pardo V: The clinical spectrum of renal disease associated with human immunodeficiency virus. *Am J Kid Dis* 12:131–137, 1988
7. Humphreys MH, Schoenfield PY: Renal complications in patients with the acquired immune deficiency syndrome (AIDS). *Am J Nephrol* 7:1–7, 1987
8. Vaziri ND, Barbari A, Licorish K, Cesario T, Gupta S: Spectrum of renal abnormalities in acquired immune-deficiency syndrome. *J Natl Med Assoc* 77:369–375, 1985
9. Thung SN, Gerber MA, Benkov KJ, Guttenberg M, Gordon RE: Chronic active hepatitis in a child with human immunodeficiency virus infection. *Arch Pathol Lab Med* 112:914–916, 1988
10. Cantor ES, Kimmel PL, Bosch JP: Effect of race on expression of acquired immunodeficiency syndrome-associated nephropathy. *Arch Int Med* 151:125–128, 1991
11. Kimmel PL, Phillips TM, Ferreira Centeno A, Farkas Szallasi T, Abraham AA, Garrett CT: Brief report: Idiotypic IgA nephropathy in patients with human immunodeficiency virus infection. *N Engl J Med* 327:702–706, 1992
12. Dickie P, Felser J, Eckhaus M, Bryant J, Silver J, Marinos N, Notkins AL: HIV-associated nephropathy in transgenic mice expressing HIV-1 genes. *Virology* 185:109–119, 1991
13. Kopp JB, Klotman ME, Adler SH, Bruggeman LA, Eckhaus M, Dickie P, Marinos NJ, Bryant JL, Notkins AL, Klotman PE: Progressive glomerulosclerosis and enhanced renal accumulation of basement membrane components in mice transgenic for HIV-1 genes. *Proc Natl Acad Sci USA* 89:1577–1581, 1992
14. Delli Bovi P, Curatola AM, Kern FG, Greco A, Ittmann M, Basilico C: An oncogene isolated by transfection of Kaposi's sarcoma DNA encodes a growth factor that is a member of the FGF family. *Cell* 50:729–737, 1987
15. Huang YQ, Li JJ, Moscatelli D, Basilico C, Nicolaides A, Zhang WG, Poiesz BJ, Friedman Kien AE: Expression of int-2 oncogene in Kaposi's sarcoma lesions. *J Clin Invest* 91:1191–1197, 1993
16. Schulze Osthoff K, Goerdt S, Sorg C: Expression of basic fibroblast growth factor (bFGF) in Kaposi's sarcoma: an immunohistologic study. *J Invest Dermatol* 95:238–240, 1990
17. Xerri L, Hassoun J, Planche J, Guigou V, Grob JJ, Parc P, Birnbaum D, Delapeyriere O: Fibroblast growth factor gene expression in AIDS-Kaposi's sarcoma detected by in situ hybridization. *Am J Pathol* 138:9–15, 1991
18. Gonzalez AM, Buscaglia M, Ong M, Baird A: Distribution of basic fibroblast growth factor in the 18-day rat fetus: Localization in the basement membranes of diverse tissues. *J Cell Biol* 110:753–765, 1990
19. Baird A, Esch F, Bohlen P, Ling N, Gospodarowicz D: Isolation and partial characterization of an endothelial cell growth factor from the bovine kidney: Homology with basic fibroblast growth factor. *Regul Pept* 12:201–213, 1985
20. Risau W, Ekblom P: Production of a heparin-binding angiogenesis factor by the embryonic kidney. *J Cell Biol* 103:1101–1107, 1986
21. Gospodarowicz D, Ferrara N, Schweigerer L, Neufeld G: Structural characterization and biological functions of fibroblast growth factor. *Endocr Rev* 8:95–114, 1987
22. Lindner V, Reidy MA: Proliferation of smooth muscle cells after vascular injury is inhibited by an antibody against basic fibroblast growth factor. *Proc Natl Acad Sci USA* 88:3739–3743, 1991
23. Takeuchi A, Yoshizawa N, Yamamoto M, Sawasaki Y, Oda T, Senoo A, Niwa H, Fuse Y: Basic fibroblast growth factor promotes proliferation of rat glomerular visceral epithelial cells in vitro. *Am J Pathol* 141:107–116, 1992
24. Zhang GH, Ichimura T, Wallin A, Kan M, Stevens JL: Regulation of rat proximal tubule epithelial cell growth by fibroblast growth factors, insulin-like growth factor-1 and transforming growth factor-beta, and analysis of fibroblast growth factors in rat kidney. *J Cell Physiol* 148:295–305, 1991
25. Floege J, Eng E, Lindner V, Alpers CE, Young BA, Reidy MA, Johnson RJ: Rat glomerular mesangial cells synthesize basic fibroblast growth factor. Release, upregulated synthesis, and mitogenicity in mesangial proliferative glomerulonephritis. *J Clin Invest* 90:2362–2369, 1992
26. Eguchi J, Nomata K, Kanda S, Igawa T, Taide M, Koga S, Matsuya F, Kanetake H, Saito Y: Gene expression and immunohistochemical localization of basic fibroblast growth factor in renal cell carcinoma. *Biochem Biophys Res Commun* 183:937–944, 1992
27. Fujimoto K, Ichimori Y, Kakizoe T, Okajima E, Sakamoto H, Sugimura T, Terada M: Increased serum levels of basic fibroblast growth factor in patients with renal cell carcinoma. *Biochem Biophys Res Commun* 180:386–392, 1991
28. Nguyen M, Watanabe H, Budson AE, Richie JP, Folkman J: Elevated levels of the angiogenic peptide basic fibroblast growth factor in urine of bladder cancer patients. *J Natl Cancer Inst* 85:241–242, 1993
29. Zamenhof S, Van Marthens E, Margolis FL: DNA (cell number) and protein in neonatal brain: alteration by maternal dietary protein restriction. *Science* 160:322–323, 1968
30. Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–254, 1976
31. Chirgwin JM, Przybyla AE, Macdonald RJ, Rutter WJ: Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* 18:5294–5299, 1979
32. Ahmad N, Venkatesan S: Nef protein of HIV-1 is a transcriptional repressor of HIV-1 LTR. *Science* 241:1481–1485, 1988
33. Abraham JA, Whang JL, Tumolo A, Mergia A, Friedman J, Gospodarowicz D, Fiddes JC: Human basic fibroblast growth factor: Nucleotide sequence and genomic organization. *EMBO J* 5:2523–2528, 1986

34. RAY PE, CASTREN E, RULEY EJ, SAAVEDRA JM: Different effects of sodium or chloride depletion on angiotensin II receptors in rats. *Am J Physiol* 258:R1008–R1015, 1990
35. JEANNY JC, FAYEIN N, MOENNER M, CHEVALLIER B, BARRITAULT D, COURTOIS Y: Specific fixation of bovine brain and retinal acidic and basic fibroblast growth factors to mouse embryonic eye basement membranes. *Exp Cell Res* 171:63–75, 1987
36. MOSCATELLI D: High and low affinity binding sites for basic fibroblast growth factor on cultured cells: Absence of a role for low affinity binding in the stimulation of plasminogen activator production by bovine capillary endothelial cells. *J Cell Phys* 131:123–130, 1987
37. TAUB M, SATO G: Growth of functional primary cultures of kidney epithelial cells in defined medium. *J Cell Phys* 105:369–378, 1980
38. RODEMANN HP, MULLER GA, KNECHT A, NORMAN JT, FINE LG: Fibroblasts of rabbit kidney in culture. I. Characterization and identification of cell-specific markers. *Am J Physiol* 261:F283–F291, 1991
39. KANDEL J, BOSSY WETZEL E, RADVANYI F, KLAGSBRUN M, FOLKMAN J, HANAHAN D: Neovascularization is associated with a switch to the export of bFGF in the multistep development of fibrosarcoma. *Cell* 66:1095–1104, 1991
40. GRANT DS, TASHIRO K, SEGUI REAL B, YAMADA Y, MARTIN GR, KLEINMAN HK: Two different laminin domains mediate the differentiation of human endothelial cells into capillary-like structures *in vitro*. *Cell* 58:933–943, 1989
41. RAY PE, AGUILERA G, KOPP JB, HORIKOSHI S, KLOTMAN PE: Angiotensin II receptor-mediated proliferation of cultured human fetal mesangial cells. *Kidney Int* 40:764–771, 1991
42. TAUB M, WANG Y, SZCZESNY TM, KLEINMAN HK: Epidermal growth factor or transforming growth factor alpha is required for kidney tubulogenesis in matrigel cultures in serum-free medium. *Proc Natl Acad Sci USA* 87:4002–4006, 1990
43. MUTHUKRISHNAN L, WARDER E, MCNEIL PL: Basic fibroblast growth factor is efficiently released from a cytosolic storage site through plasma membrane disruptions of endothelial cells. *J Cell Physiol* 148:1–16, 1991
44. ZHANG G, ICHIMURA T, MAIER JA, MACIAG T, STEVENS JL: A role for fibroblast growth factor type-1 in nephrogenic repair. Autocrine expression in rat kidney proximal tubule epithelial cells in vitro and in the regenerating epithelium following nephrotoxic damage by S-(1,1,2,2-tetrafluoroethyl)-L-cysteine in vivo. *J Biol Chem* 268:11542–11547, 1993
45. WHALEN GF, SHING Y, FOLKMAN J: The fate of intravenously administered bFGF and the effect of heparin. *Growth Factors* 1:157–164, 1989
46. STOW JL, SOROKA CJ, MACKAY K, STRIKER L, STRIKER G, FARQUHAR MG: Basement membrane heparan sulfate proteoglycan is the main proteoglycan synthesized by glomerular epithelial cells in culture. *Am J Pathol* 135:637–646, 1989
47. YAYON A, KLAGSBRUN M, ESKO JD, LEDER P, ORNITZ DM: Cell surface, heparin-like molecules are required for binding of basic fibroblast growth factor to its high affinity receptor. *Cell* 64:841–848, 1991
48. KLAGSBRUN M, BAIRD A: A dual receptor system is required for basic fibroblast growth factor activity. *Cell* 67:229–231, 1991
49. ADLER S, ENG B: Reversal of inhibition of rat glomerular epithelial cell growth by growth factors. *Am J Pathol* 136:557–563, 1990
50. GROGGEL GC, MARINIDES GN, HOVINGH P, HAMMOND E, LINKER A: Inhibition of rat mesangial cell growth by heparan sulfate. *Am J Physiol* 258:F259–F265, 1990
51. CASTELLOT JJ JR, HOOVER RL, HARPER PA, KARNOVSKY MJ: Heparin and glomerular epithelial cell-secreted heparin-like species inhibit mesangial-cell proliferation. *Am J Pathol* 120:427–435, 1985