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Tubular cell phenotype in HIV-associated nephropathy: Role of phospholipid lysophosphatidic acid

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ABSTRACT

Collapsing glomerulopathy and microcysts are characteristic histological features of HIV-associated nephropathy (HIVAN). We have previously reported the role of epithelial mesenchymal transition (EMT) in the development of glomerular and tubular cell phenotypes in HIVAN. Since persistent tubular cell activation of NFκB has been reported in HIVAN, we now hypothesize that HIV may be contributing to tubular cell phenotype via lysophosphatidic acid (LPA) mediated downstream signaling. Interestingly, LPA and its receptors have also been implicated in the tubular interstitial cell fibrosis (TIF) and cyst formation in autosomal dominant polycystic kidney disease (PKD). Primary human proximal tubular cells (HRPTCs) were transduced with either empty vector (EV/HRPTCs), HIV (HIV/HRPTCs) or treated with LPA (LPA/HRPTC). Immunoelectrophoresis of HIV/HRPTCs and LPA/HRPTCs displayed enhanced expression of pro-fibrotic markers: a) fibronectin (2.25 fold), b) connective tissue growth factor (CTGF; 4.8 fold), c) α-smooth muscle actin (α-SMA; 12 fold), and d) collagen I (5.7 fold). HIV enhanced tubular cell phosphorylation of ILK-1, FAK, PI3K, Akt, ERKs and P38 MAPK. HIV increased tubular cell transcriptional binding activity of NF-κB; whereas, a LPA biosynthesis inhibitor (AACOCF3), a DAG kinase inhibitor, a LPA receptor blocker (Ki16425), a NF-κB inhibitor (PDTC) and NFκB-siRNA not only displayed downregulation of a NFκB activity but also showed attenuated expression of profibrotic/EMT genes in HIV milieu. These findings suggest that LPA could be contributing to HIV-induced tubular cell phenotype via NFκB activation in HIVAN.

1. Introduction

Renal tubular epithelial cell infection and microcyst formation have been reported in HIV associated nephropathy (HIVAN). It has been suggested that HIV-1 gene transcript increases as tubules dilate and the epithelium becomes flattened and atrophic (Rao, 1996; Ross et al., 2001; Salhan et al., 2012). To date, the fate of the segments that become infected by HIV-1 and the ones which develop microcysts in HIVAN is not clearly understood. The molecular mechanism by which the viral gene expression leads to microcyst formation is far from clear. We have earlier demonstrated the role of epithelial mesenchymal transition for proliferative phenotype for both glomerular and tubular cells in HIVAN (Kumar et al., 2011; Rai et al., 2013; Yadav et al., 2010).

Renal fibrosis is characterized by glomerulosclerosis and tubulointerstitial fibrosis (TIF) with accumulation of extracellular matrix (collagens and fibronectin) and infiltration of macrophages and lymphocytes (Salhan et al., 2012; Yadav et al., 2010; Yokoi et al., 2002). During the last decade, connective tissue growth factor (CTGF) has been implicated to have a dominant role either directly or indirectly in mediating tubular cell injury (Kumar et al., 2011; Yadav et al., 2010; Yokoi et al., 2002). EMT plays a critical role in renal fibrosis. Tightly bound epithelial cells get detached following the loss of cell polarity and get converted to mesenchymal cells expressing mesenchymal proteins and develop migratory potential. In the kidney and lung, collagen and CTGF are excessively expressed. CTGF is a secreted matricellular protein which plays an important role in the pathogenesis of chronic fibrotic diseases.

Recent reports demonstrate significant involvement of phospholipids in the development of fibrosis including platelet activating factor (PAF), phosphatidyl choline, and lysophosphatidic acid (LPA) (Gonzalez et al., 2008; Natoli et al., 2010). Cystic fluid in patients of polycystic

Abbreviations: HRPTC, human renal proximal tubular cells; PL A 1/2, phospholipase A 1/2; LysoPLD, lysophospholipase D; PDTC, pyrrolidine dithiocarbamate; DAG, di-acyl glycerol; CKD, chronic kidney disease; ESRD, end stage renal disease (ESRD); ECM, extracellular matrix; TIF, tubular interstitial fibrosis.

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kidney disease (PKD) has been shown to have increased levels of LPA (Blazer-Yost et al., 2011). LPA seeps to vascular as well as interstitial spaces and promotes cyst enlargement. LPA is known to regulate several cellular processes including cell motility, proliferation, survival, and differentiation (Blazer-Yost et al., 2011; Yang et al., 2005). LPA acts via specific G-protein coupled receptors (LPA1 to LPA5) (Yang et al., 2005). However, the metabolic origin of LPA remains to be elucidated. Based on biosynthetic pathways, several enzymes involved seem to be activated such as phospholipases A1/A2, lysophospholipase D/autotoxin (ATX), glycerol phosphate acyl transferase, or monoacyl glycerol kinase (MAGK)—all leading to increased LPA synthesis (Ye and Chun, 2010).

In the present study, we have demonstrated that HIV-transduced/LPA treated-HRPTCs display an increased expression of molecular markers of profibrotic/EMT phenotype; moreover, HIV- and LPA-induced downstream signaling events are ILK–FAK dependent and result in NFκB activation and gene transcription.

2. Methods

2.1. Cells and viruses

Human renal proximal tubular cells (HRPTCs) were obtained from ScienCell Research Labs (Carlsbad, CA, USA). HRPTCs were cultured using EpiCM medium (ScienCell). Cells were maintained at 37 °C, 5% CO_2 in a humidified incubator. LPA was purchased from Avanti Polar Lipids (Alabaster, AL). LPA was prepared in PBS containing 0.1% BSA (vol/vol) and sonicated before use. HRPTCs, control (C/HRPTCs) and empty vector (EV/HRPTC) or treated with HIV/LPA [(HIV/HRPTC)/(LPA/HRPTCs)] wherever indicated were used in the study.

2.2. Production of pseudotyped retroviral supernatant

Replication-defective viral supernatants were prepared as published previously (Salhan et al., 2012). Briefly, green fluorescence protein (GFP) reporter gene (from pEGFP-C1; Clontech, Palo Alto, CA) was substituted in place of *gag/pol* genes in HIV-1 proviral construct pNL4-3. This parental construct (pNL4-3: ΔG/P-GFP) was used to produce VSV.G pseudotyped viruses to provide pleiotropism and high-titer virus stocks. Infectious viral supernatants were produced by the transient transfection of 293T cells using Effectene (Qiagen, Valencia, CA) according to the manufacturer's instructions. The HIV-1 *gag/pol* and VSV.G envelope genes were provided in *trans* using pCMV R8.91 and pMD.G plasmids (gifts by Dr. Didier Trono, Salk Institute, La Jolla, CA). Viral stocks ranging from 10^5 to 10^6 GEU/ml were obtained.

2.3. Transfection

HRPTCs were transfected using Lipofectamine Plus reagent according to the manufacturer's protocol with a total of 1 μg/well of plasmid DNA. Twenty-four hours later, the cells were treated with HIV or LPA (24 h), followed by further incubation at 37 °C. For NFκB-luciferase activity, HRPTCs were transfected with NFκB-luciferase reporter plasmid and/or using DN-p65 plasmid with pCMV-β-gal by Lipofectamine Plus. pcDNA3 was used to normalize all groups to equal amounts of DNA luciferase (Promega, Madison, WI) further normalizing with β-galactosidase activity. NFκB-luciferase, DN-p65 plasmids were kindly provided by Dr. George Rawadi (Institute Pasteur, Laboratoire des Mycoplasmes, Paris, France) (Ayasolla et al., 2005). The expression vector for flag-IKKα was a gift from Dr Zheng-Gang Liu (National Institutes of Health, Bethesda, MD).

2.4. Silencing of NFκB

HRPTCs were transfected with 25–50 nM NFκB small interfering (Si) RNA (Santa-Cruz Biotechnology; Santa Cruz, CA) with Siport Neofax transfection reagent and left in optiMEM medium for 24–48 h and the cells were transferred back to HRPTC medium an hour before transfection with NL4-3 GFP.

2.5. Immunodetection by Western blot

HRPTCs, HIV/HRPTCs, and EV/HRPTCs were incubated in medium for 3 days. Cells were lysed in RIPA buffer containing 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.25% deoxycholate, 0.1% SDS, 1× protease inhibitor cocktail I (Calbiochem, EMD Biosciences, Gibbstan, NJ), 1 mM PMSF, and 0.2 mM sodium orthovanadate. Protein concentration was determined using the Biorad Protein Assay (Pierce, Rockford, IL). Protein lysates (20 μg) were separated on 12% polyacrylamide gels (PAGE, Bio-Rad, Hercules, CA) and transferred onto a nitrocellulose membrane using Bio-Rad miniblot apparatus. Nitrocellulose membranes were then subjected to immunostaining with primary antibodies against CTGF, TGF-β, fibronectin, vimentin, α-SMA and SNAIL (Santa Cruz Biotechnology, Dallas, TX, USA), NFκB pathway proteins (phosphospecific, Cell Signaling, Danvers, MA), p-ILK1, and p-FAK (EMD Millipore, Billerica, MA, USA), and subsequently with horseradish peroxidase-labeled appropriate secondary antibodies (Biorad, Hercules, CA). The blots were developed using a chemiluminescence detection kit (ThermoScientific, Rockford, IL, USA) and exposed to X-ray film (Eastman Kodak, Rochester, NY). Equal protein loading was confirmed by stripping and reprobed the same blots immunoblotting for β-actin protein. For quantification, the immunoblots were scanned, and densitometry was performed by ImageJ analysis; values were normalized to β-actin expression and expressed as fold increase when compared to control values as shown.

2.6. Preparation of nuclear extracts and electrophoretic mobility shift assay (EMSA)

Nuclear extracts from control and experimental cells (1×10^7) were prepared as described previously (Ayasolla et al., 2004, 2005). Aliquots (1 μg) were used for the electrophoretic mobility shift assay using the NFκB DNA-binding protein detection system kit (Affymetrix). Briefly, the protein-binding biotinylated DNA probes (NFκB) were incubated with nuclear extracts prepared from control and experimental cells according to the manufacturer's protocol (Panomics, Redwood City, CA). The DNA–protein binding reactions were performed at room temperature for 10 min in 10 mM Tris–HCl pH 7.9, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, and 1 mM dithiothreitol plus 1 μg of poly(dI-dC), 5% (v/v) glycerol, and ~10 ng of biotinylated NFκB probe. Protein–DNA complexes were resolved from protein-free DNA on 6% polyacrylamide gels (Invitrogen) at 4 °C in 50 mM Tris, pH 8.3, 2 mM EDTA. DNA–protein complexes and rest of the gel contents were transferred to Biodyne B membrane (Pall, Ann Arbor, MI) for 60 min at 300 mA. The membranes now containing the DNA–protein complexes were UV cross linked and chemiluminescent detection of biotinylated DNA was performed using the Panomics EMSA kit.

3. Results

3.1. HIV/HRPTCs and LPA/HRPTCs show higher expression of fibronectin, CTGF, collagen I, α-SMA, and vimentin

HIV expressing tubular cells and LPA-treated tubular cells displayed similar molecular phenotype (Fig. 1A). LPA inhibitors and a LPA receptor inhibitor (Ki16425) attenuated the expression of profibrotic/EMT molecular markers. HIV enhanced tubular cell fibronectin by an average 2.3 (50–100 U) fold, whereas LPA enhanced tubular cell fibronectin expression by an average 1.7 (1 μM–5 μM) fold. Similarly, HIV enhanced tubular CTGF expression by an average 4.85 fold and LPA increased tubular cell expression of CTGF by an average 5.15 fold at two different concentrations. HIV increased tubular cell collagen-I expression by an average of 5.95 fold and LPA enhanced collagen-I expression by an