mTOR plays a critical role in p53-induced oxidative kidney cell injury in HIVAN

Partab Rai,1 Andrei Plagov,1 Xiqian Lan,1 Nirupama Chandel,1 Tejinder Singh,1 Rivka Lederman,1
Kamesh R. Ayasolla,1 Peter W. Mathieson,2 Moin A. Saleem,2 Mohammad Husain,1 Ashwani Malhotra,1
Praveen N. Chander,3 and Pravin C. Singhal1

1Department of Medicine, Feinstein Institute for Medical Research, Hofstra North Shore LIJ Medical School, Great Neck, New York; 2Department of Pathology, New York Medical College, New York, New York, and 3University of Bristol, Bristol, United Kingdom

Submitted 6 March 2013; accepted in final form 8 May 2013

Rai P, Plagov A, Lan X, Chandel N, Singh T, Lederman R, Ayasolla KR, Mathieson PW, Saleem MA, Husain M, Malhotra A, Chander PN, Singhal PC. mTOR plays a critical role in p53-induced oxidative kidney cell injury in HIVAN. Am J Physiol Renal Physiol 305: F343–F354, 2013. First published May 15, 2013; doi:10.1152/ajprenal.00135.2013.—Oxidative stress has been implicated to contribute to HIV-induced kidney cell injury; however, the role of p53, a modulator of oxidative stress, has not been evaluated in the development of HIV-associated nephropathy (HIVAN). We hypothesized that mammalian target of rapamycin (mTOR) may be critical for the induction of p53-mediated oxidative kidney cell injury in HIVAN. To test our hypothesis, we evaluated the effect of an mTOR inhibitor, rapamycin, on kidney cell p53 expression, downstream signaling, and kidney cell injury in both in vivo and in vitro studies. Inhibition of the mTOR pathway resulted in downregulation of renal tissue p53 expression, associated downstream signaling, and decreased number of sclerosed glomeruli, tubular microcysts, and apoptosed and 8-hydroxy deoxyguanosine (8-OHdG)-positive (+ve) cells in Tg26 mice. mTOR inhibition not only attenuated kidney cell expression of p66ShcA and phospho-p66ShcA but also reactivated the redox-sensitive stress response program in the form of enhanced expression of manganese superoxide dismutase (MnSOD) and catalase. In in vitro studies, the mTOR inhibitor also provided protection against HIV-induced podocyte apoptosis. Moreover, mTOR inhibition downregulated HIV-induced podocyte (HP/HIV) p53 expression. Since HP/HIV silenced for mTOR displayed a lack of expression of p53 as well as attenuated podocyte apoptosis, this suggests that mTOR is critical for kidney cell p53 activation and associated oxidative kidney cell injury in the HIV milieu.

apoptosis; mTOR; oxidative stress; rapamycin; renal tubular cells

HIV-ASSOCIATED NEPHROPATHY is the manifestation of specific genetic (Apol1), environmental (HIV infection), and host factors (2, 10, 17, 34, 45). On that account, HIV-associated nephropathy (HIVAN) patients are being treated with highly active antiretroviral therapy (HAART). Since the advent of HAART, there has been a significant decrease in the number of HIVAN patients (2). Nonetheless, in a recent report, 50% of HIVAN patients progressed to end-stage kidney disease (ESKD) despite being on HAART (4). Therefore, treatment of HIVAN still poses a challenge. Patients with HIV infection display an imbalance in oxidative stress response in the form of lower levels of antioxidants—ascorbic acid, tocopherols, and selenium (7, 11, 13)—and higher levels of malondialdehyde and hydroperoxides (14, 40). Induction of oxidative stress contributes to HIV disease pathogenesis in multiple ways: enhanced viral replication (31, 42), increased inflammatory immune response (15), and accelerated loss of immune function (44). The role of oxidative stress in the development of HIVAN is also being increasingly recognized (20, 38, 39).

We previously reported (38, 39) the role of p66ShcA pathway in the deactivation of the redox-sensitive redox pathway in HIV in both podocytes and tubular cells. p53 has been shown to be upstream of p66ShcA signaling and is known to activate the promoter of p66ShcA (47); p53-null mice displayed attenuated p66ShcA expression and diminished reactive oxygen species (ROS) generation (47). Interestingly, downregulation of p66ShcA in this model was not accompanied with enhanced longevity; on the contrary, these mice died prematurely because of enhanced carcinogenesis (47). Unlike p53-null mice, p66ShcA-knockout mice not only displayed attenuated ROS generation but also had a longer life span (29, 32). It has been suggested that p53 only induced ROS generation signaling through p66ShcA (47), whereas its downstream signaling pertaining to tumor suppression was not mediated through p66ShcA pathway. We asked whether activation of p53 in HIVAN was also associated with activation of p66ShcA pathway.

The mammalian target of rapamycin (mTOR) pathway plays an important role in the development and progression of HIVAN (25). We have previously demonstrated (25, 35) that kidney cells displayed enhanced expression of phospho-mTOR in both HIVAN mice and HIVAN patients. In vivo studies, inhibition of mTOR pathway slowed down the progression of HIVAN, whereas in vitro studies inhibition of mTOR pathway prevented HIV-induced tubular cell growth. Recently, we demonstrated (20, 38, 39) the role of oxidative stress in the development of HIV-induced kidney cell injury. We now hypothesize that mTOR pathway may be modulating HIV-induced kidney cell injury by down-regulating p53-mediated oxidative stress.

p53 plays a central role in responding to DNA damage and inhibits mTOR pathway in stressed cells (18). p53 inhibits mTOR by activation of AMP-activated protein kinase (AMPK) and subsequent regulation of the TSC1/TSC2 complex (21). Inactivation of either TSC1/TSC2 complex or AMPK inhibits the impact of p53 on mTOR pathway (21). Through inhibition of mTOR, p53 could regulate inhibition of translation and activation of autophagy; thus communication between p53 and mTOR pathway may be contributing to normal cell growth and...
proliferation. Conversely, HIV promotes lymphocyte apoptosis through mTOR-mediated activation of p53 pathway (8, 9).

In the present study, we evaluated the effect of inhibition of the mTOR pathway on renal tissue p53 expression and associated downstream signaling in HIVAN (Tg26) mice. In addition, we studied the contributory role of mTOR-mediated p53-induced kidney cell injury.

**MATERIALS AND METHODS**

**HIV transgenic (Tg26) mice.** We used age- and sex-matched FVB/N (control) and Tg26 (with FVB/N background) mice. Breeding pairs of FVB/N mice were obtained from Jackson Laboratories (Bar Harbor, ME). Breeding pairs to develop Tg26 colonies were kindly gifted by Dr. Paul E. Klotman, President and CEO, Baylor College of Medicine (Houston, TX). Tg26 transgenic animals have the proviral transgene pNL4-3:d1443, which encodes all the HIV-1 genes except gag and pol, rendering the mice noninfectious.

The Ethics Review Committee for Animal Experimentation of Long Island Jewish Medical Center approved the experimental protocol.

**Experimental studies.** Three-week-old Tg26 and FVBN (control) mice (n = 6) were administered either normal saline (Tg26) or rapamycin (5 mg/kg ip every other day; Tg26/Rapa) for 8 wk and then were anesthetized and killed. Both kidneys were excised; one was processed for histological studies, while the other was used for RNA and protein extraction. Three-micrometer sections were prepared and stained with hematoxylin-eosin and periodic acid-Schiff (PAS). Renal sections were coded and examined under light microscopy. Twenty random fields (n = 20) per mouse were examined to score percentage of the involved glomeruli and tubules. Glomerular lesions were classified as segmental glomerulosclerosis (SGS), global glomerulosclerosis (GGS), and collapsing glomerulosclerosis (CGS). Severity of tubular injury was scored in the form of tubular dilatation (% of dilated tubules/section) and size of microcysts (1 to 4) by two investigators who were unaware of the experimental conditions.

**Preparation of podocytes.** Human podocytes were prepared and transduced with HIV (NL4-3) as described previously (16).

**Table 1. Primer sequences**

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
<th>Reference Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catalase F</td>
<td>GCTGAAAGTCTACAGATG</td>
<td>NM_009804.2</td>
</tr>
<tr>
<td>R</td>
<td>GTCATCGACATGTCGATG</td>
<td></td>
</tr>
<tr>
<td>MnSOD F</td>
<td>GACCTGTCCTCCTATGAAAGC</td>
<td>NM_013671.3</td>
</tr>
<tr>
<td>R</td>
<td>GACCTGTCCTCCTATGAAAGC</td>
<td></td>
</tr>
<tr>
<td>P53 F</td>
<td>GCAATCAAGACCTGCACACTG</td>
<td>NM_011640.3</td>
</tr>
<tr>
<td>R</td>
<td>GCCACATGGTACGCTGCTTTG</td>
<td></td>
</tr>
<tr>
<td>P21 F</td>
<td>CTGCTCTGAGTCTGCTTGACT</td>
<td>NM_001111099.1</td>
</tr>
<tr>
<td>R</td>
<td>TCTCTTGGAGAAGACACAATG</td>
<td></td>
</tr>
<tr>
<td>P27 F</td>
<td>AGGAGAGCGCAAGATGCGAG</td>
<td>NM_009875.4</td>
</tr>
<tr>
<td>R</td>
<td>CAGAGTTTGGCTGGAAGCCCAAA</td>
<td></td>
</tr>
<tr>
<td>Fas-L F</td>
<td>CAGAGTTTGGCTGGAAGCCCAAA</td>
<td>NM_010177.4</td>
</tr>
<tr>
<td>R</td>
<td>AGATTCCCTAAAAATGGACAGA</td>
<td></td>
</tr>
<tr>
<td>GADD45 F</td>
<td>AUATGAGCAGTGGAGAATTCC</td>
<td>NM_007836.1</td>
</tr>
<tr>
<td>R</td>
<td>TTTCGAGCTAGTGGAGAATTCC</td>
<td></td>
</tr>
<tr>
<td>P53 (human) F</td>
<td>GTTCGAGCGACATGGAAGG</td>
<td>M60950</td>
</tr>
<tr>
<td>R</td>
<td>CTCTACCTGCTGCTGGCTT</td>
<td></td>
</tr>
<tr>
<td>MnSOD (human) F</td>
<td>AGGTGAATGTTGGTGTGCTTCA</td>
<td>NG_008729.1</td>
</tr>
<tr>
<td>R</td>
<td>CAATCCCTGACAGCTGGAAT</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 1. Tg26 mice display kidney cell oxidative damage, apoptosis, and HIV-associated nephropathy (HIVAN) phenotype. Renal cortical sections of control and Tg26 mice were stained with periodic acid-Schiff (PAS) and immunolabeled for 8-hydroxy deoxyguanosine (8-OHdG) and TdT-mediated dUTP nick end labeling (TUNEL). Representative microphotographs are shown. A: renal cortical section from a control mouse displaying 8-OHdG+ve cells (darkly stained nuclei). B: renal cortical section from a Tg26 mouse displaying enhanced number of 8-OHdG+ve cells. C: renal cortical section from a control mouse stained for TUNEL. D: renal cortical section from a Tg26 mouse displaying TUNEL+ve cells (indicated by arrows). E: renal cortical section from a control mouse displaying a normal glomerulus and tubules. F: renal cortical section from a Tg26 mouse displaying a sclerosed glomerulus (indicated by arrow) and a microcyst (indicated by arrowhead).
TUNEL assay. TdT-mediated dUTP nick end labeling (TUNEL) assay was performed with the Apoptosis Detection Tacs TdT Kit (R&D Systems, Minneapolis, MN) as described previously (16).

Immunohistochemical studies. Renal cortical sections from control and HIV-1 transgenic (Tg26) mice were deparaffinized and immunolabeled for 8-hydroxy deoxyguanosine (8-OHdG), phospho-p66ShcA, and TUNEL as described previously (20, 38, 39).

Silencing of mTOR/p53. HIV-transduced podocytes (HP/HIV) were transfected with 25 nM mTOR/p53 small interfering RNA (siRNA) (Cell Signaling) with Siport Neofax transfection reagent and suspended in optiMEM medium for 24 h. Control and transfected cells were used under control and experimental conditions.

Western blotting studies. Control and experimental cells or renal tissues were lysed, and Western blots were prepared and probed for p53, p66ShcA (total), phospho-p66ShcA, p21, p27, GADD45, and Fas. Protein transfer on nitrocellulose blots were checked by Ponceau Red dye. Equal protein loading was confirmed by stripping and probing the same blots for actin.

Reverse transcription PCR analysis. Control (HP; transduced with empty vector) and experimental (HP/HIV) cells and renal tissues from Tg26 and Tg26/Rapa mice were used to quantify mRNA expression of p53, p21, p27, GADD45, manganese superoxide dismutase (MnSOD), FasL, and catalase. RNA was extracted with TRizol (Invitrogen). For cDNA synthesis, 2 μg of the total RNA was preincubated with 2 nmol of random hexamer (Invitrogen) at 65°C for 5 min. Subsequently, 8 μl of the reverse transcription (RT) reaction mixture containing cloned AMV RT, 0.5 mmol each of the mixed nucleotides, 0.01 mol of dithiothreitol, and 1,000 U/ml RNaseOUT (Invitrogen) containing cloned AMV RT, 0.5 mmol each of the mixed nucleotides, 0.01 mol of dithiothreitol, and 1,000 U/ml RNaseOUT (Invitrogen) were incubated at 42°C for 50 min. For a negative control, a reaction mixture without RNA or RT was used. Samples were subsequently incubated at 85°C for 5 min to inactivate the RT.

Quantitative PCR was carried out in an ABI Prism 7900HT by 10.220.33.1 on October 21, 2017 http://ajprenal.physiology.org/ Downloaded from

RESULTS

Tg26 mice display kidney cell oxidative damage, apoptosis, and HIVAN phenotype. Renal cortical sections of control and Tg26 mice were immunolabeled for 8-OHdG and TUNEL assay. Representative microphotographs are shown in Fig. 1. Tg26 mice displayed an enhanced number of 8-OHdG-positive (+ve) cells (Fig. 1B) as well as an increased number of apoptosed kidney cells/field (Fig. 1D) compared with control mice (Fig. 1, A and C). Renal cortical sections of control and Tg26 mice were also stained with PAS and evaluated for renal histology. Representative microphotographs of renal cortical sections of a control and a Tg26 mouse are shown in Fig. 1, E and F, respectively. The Tg26 mouse displayed a sclerosed glomerulus and a tubular microcyst.

SYBR green was used as a detector and ROX as a stabilizing dye. Results (means ± SD) represent numbers of cell samples or animals as described in figures legend. The data were analyzed with the comparative Ct method (ΔΔCt, where Ct is threshold cycle). Differences in Ct are used to quantify the relative amount of PCR target contained within each well. The data were expressed as relative mRNA expression in reference to control, normalized to quantity of RNA input by performing measurements on an endogenous reference gene, GAPDH.

Statistical analysis. For comparison of mean values between two groups, the unpaired t-test was used. To compare values between multiple groups, analysis of variance (ANOVA) and a Bonferroni multiple-range test were used to calculate the P value. Statistical significance was defined as P < 0.05. All values are displayed as means ± SD.

RENAL CYTOLOGY: CONTROL AND Tg26 MICE

Fig. 2. Renal tissues of Tg26 mice display enhanced expression of p53 and downstream signaling molecules. Cellular lysates of renal tissues of control and Tg26 mice were electrophoresed and probed for p53, p21, p27, GADD45, and Fas. The same blots were reprobed for actin. A: representative gels displaying renal tissue expression of p53 from 2 control (FVBN) and 2 Tg26 mice. B: densitometric data (n = 3). **P < 0.01 compared with FVBN. C: representative gels displaying renal tissue expression of p21 from 2 control (FVBN) and 2 Tg26 mice. D: densitometric data (n = 3). **P < 0.01 compared with FVBN. E: representative gels displaying renal tissue expression of p27 from 2 control (FVBN) and 2 Tg26 mice. F: cumulative data (n = 3). **P < 0.01 vs. FVBN. G: representative gels displaying renal tissue expression of GADD45 from 2 control (FVBN) and 2 Tg26 mice. H: cumulative data in bar graphs (n = 3). **P < 0.05 vs. FVBN. I: representative gels displaying renal tissue expression of Fas from 2 control (FVBN) and 2 Tg26 mice. J: densitometric analysis (n = 3). **P < 0.01 vs. FVBN.
Renal tissues of Tg26 mice display enhanced expression of p53 and downstream signaling molecules. Cellular lysates of renal tissues of control and Tg26 mice were electrophoresed and probed for p53, p21, p27, GADD45, and Fas. The same blots were reprobed for actin. Representative gels displaying renal tissues from two different mice are shown in Fig. 2. Cumulative data are shown in bar graphs (n = 3). Renal tissues of Tg26 mice displayed enhanced expression of p53 (Fig. 2, A and B). Similarly, renal tissues of Tg26 mice displayed enhanced expression of p21 (Fig. 2, C and D), p27 (Fig. 2, E and F), GADD45 (Fig. 2, G and H), and Fas (Fig. 2, I and J) compared with respective control mice. These findings indicate that there is an activation of p53-mediated downstream signaling in renal tissues of HIVAN mice.

mTOR inhibition attenuates HIV-induced kidney cell expression of p66shcA in HIVAN mice. We previously reported (20, 39) the role of p66Shc pathway in HIV-induced oxidative kidney cell injury in both in vitro and in vivo studies. To determine the role of mTOR in HIV-induced kidney cell injury, renal cortical sections of control, Tg26, and Tg26/Rapa mice were immunolabeled for p66ShcA (total) and phospho-p66ShcA. Representative microphotographs are shown in Fig. 3A. Tubular cells in Tg26 mice displayed enhanced expression of both p66ShcA and phospho-p66ShcA; however, renal cortical sections of control and Tg26/Rapa mice revealed minimal (background) expression of phospho-p66ShcA. To quantify renal tissue p66ShcA (both total and phosphorylated fractions), preparations of renal tissue lysates from three different animals were electrophoresed and probed for total p66ShcA (Fig. 3B) and phospho-p66ShcA (Fig. 3C). The same blots were reprobed for actin (since HIV infection as well as mTOR inhibition altered expression of total p66ShcA, we have used actin to indicate the protein loading). Representative gels are shown in Fig. 3, B and C; cumulative data are shown in bar graphs. These findings indicated that mTOR inhibition had the potential to inhibit p66ShcA expression in HIVAN mice.

mTOR inhibition attenuates oxidative DNA damage in kidney cells in HIVAN mice. If mTOR could inhibit oxidative stress pathway in HIVAN mice, we asked whether mTOR also provided protection against HIV-induced oxidative kidney cell damage (38). To determine the effect of mTOR inhibition on oxidative DNA damage, renal cortical sections of control, Tg26, and Tg26/Rapa mice were immunolabeled for 8-OHdG. Representative microphotographs are shown in Fig. 4A. Cumulative data in the form of number of 8-OHdG+ve cells/tubule are shown in Fig. 4B. These findings indicate that mTOR inhibition provides protection against HIV-induced oxidative kidney cell DNA damage.
mTOR inhibition attenuates kidney cell apoptosis in HIVAN mice. If mTOR inhibition was associated with diminished oxidative kidney cell DNA damage in HIVAN mice, we expected a decreased number of apoptosed kidney cells in Tg26/Rapa mice. Renal cortical sections of control, Tg26, and Tg26/Rapa mice were stained for TUNEL+ve cells. Representative microphotographs are shown in Fig. 5A. Cumulative data for number of TUNEL+ve cells/field are shown in Fig. 5B (n = 3). Renal cortical sections of Tg26 mice displayed an increased number of TUNEL+ve cells/field; however, mTOR inhibition decreased the number of TUNEL+ve cells/field in Tg26/Rapa mice.

Fig. 4. mTOR inhibition attenuates oxidative DNA damage in kidney cells in HIVAN mice. Renal cortical sections of control, Tg26, and Tg26/Rapa mice were immunolabeled for 8-OHdG. A: representative microphotographs displaying 8-OHdG+ve cells (darkly stained nuclei) in cortical sections of control (FVBN), Tg26, and Tg26/Rapa mice. B: cumulative data (n = 3) for number of 8-OHdG+ve cells/tubule.

mTOR inhibition attenuates renal lesions in HIVAN mice. Renal cortical sections of control, Tg26, and Tg26/Rapa mice were evaluated for the severity of renal lesions. Representative microphotographs are shown in Fig. 6A. Cumulative data in terms of percentage of glomeruli showing sclerosis and collapsing phenotype are shown in Fig. 6B. Control mice did not display any glomerular or tubular lesions. Tg26 mice displayed sclerosed (SGS), collapsed (CGS) and globally sclerosed (GGS) glomeruli. Tg26 mice also showed tubular dilatation with microcyst formation, whereas rapamycin-receiving Tg26 mice displayed only a few sclerosed glomeruli (SGS and CGS) and none with global sclerosis (GGS). Similarly, rapamycin-
receiving mice displayed only occasional dilated tubules and significant decrease in microcyst size.

mTOR inhibition attenuates renal tissue expression of p53 and associated downstream signaling in HIVAN mice. To determine the effect of mTOR inhibition on p53 pathway, renal tissue lysates of control, Tg26, and Tg26/Rapa mice were electrophoresed and probed for p53 and reprobed for actin. Representative gels from two different mice are displayed in Fig. 7A; cumulative data in bar graphs are also shown (n = 3). Renal tissues of Tg26/Rapa mice displayed attenuated p53 expression compared with Tg26 mice.

To determine the effect of mTOR inhibition on p53-mediated downstream signaling, protein blots of renal tissues of control, Tg26, and Tg26/Rapa mice were probed for p21, p27, and Fas proteins. The same blots were reprobed for actin. Representative gels from two different mice are shown in Fig. 7B–D, respectively. Cumulative data in bar graphs (n = 3) are also shown in Fig. 7, B–D. Renal tissues of Tg26 mice displayed enhanced expression of p21, p27 and Fas, whereas mTOR inhibition attenuated expression of p21, p27, and Fas in Tg26/Rapa mice.

To determine the effect of mTOR inhibition on renal tissue transcription of p53 and associated downstream molecules, total RNAs were extracted from renal tissues of control, Tg26, and Tg26/Rapa mice (n = 3) and probed for p53, p21, p27, and GADD45. As shown in Fig. 8A, renal tissues of Tg26 mice displayed enhanced p53 mRNA expression whereas mTOR inhibition attenuated this effect in Tg26/Rapa mice. Similarly, mTOR inhibition attenuated expression of p21 (Fig. 8B), p27 (Fig. 8C), and GADD45 (Fig. 8D) in Tg26/Rapa mice.

These findings indicate that mTOR not only inhibits renal tissue transcription and protein expression of p53 but also downregulates p53-mediated downstream signaling in HIVAN mice.

mTOR inhibition enhances renal tissue expression of antioxidants and downregulates expression of proapoptotic molecules. Since mTOR inhibition downregulated renal tissue p66ShcA phosphorylation in HIVAN mice, we expected that it would also reactivate the redox-sensitive stress response program in HIVAN mice. To quantify the transcription of antioxidants, total RNA was extracted from renal tissues from control, Tg26, and Tg26/Rapa mice and probed for MnSOD and catalase by real-time PCR studies. As expected, renal tissues from Tg26 mice displayed attenuation of mRNA expression of MnSOD (Fig. 9A) and catalase (Fig. 9B). On the other hand, mTOR inhibition enhanced the expression of both MnSOD and catalase in HIVAN mice. These findings indicate that mTOR inhibition has the potential to reactivate the redox-sensitive stress response program in HIVAN mice.

To determine the effect of mTOR inhibition on HIV-induced expression of proapoptotic molecules, total RNA was extracted from renal tissues of control, Tg26, and Tg26/Rapa mice and probed for FasL (Fas ligand) by real-time PCR studies. As shown in Fig. 9C, renal tissues from Tg26 mice displayed enhanced transcription of FasL whereas mTOR inhibition attenuated FasL expression in Tg26/Rapa mice.

mTOR is critical for podocyte p53 expression. To silence mTOR in HP, HP were transfected with either scrambled siRNA (SCR) or mTOR siRNA. Protein blots of control, HP/SCR and HP/mTOR siRNA were probed for p53 and reprobed for actin. Representative gels and densitometric analysis are shown in Fig. 10A.

To determine whether mTOR is critical for podocyte p53 expression in the HIV milieu, protein blots of control, HIV-transduced HP (HP/HIV), mTOR siRNA-transfected HP/HIV, and scrambled siRNA-transfected HP/HIV were probed for P53 and reprobed for actin. Cumulative data are shown in bar graphs in Fig. 10B (n = 3). HP/HIV displayed enhanced...
expression of p53, whereas HP/HIV lacking mTOR displayed attenuated expression of p53. These findings indicate that mTOR expression is critical for HIV-induced podocyte p53 expression.

To determine the effect of mTOR inhibition on podocyte transcription of p53 in the HIV milieu, HP and HP/HIV were incubated in medium with or without rapamycin (1 ng/ml) for 48 h. Total RNA was extracted and probed for p53 (n = 3). As shown in Fig. 10C, HIV enhanced p53 mRNA expression in podocytes; however, rapamycin attenuated this effect.

To determine the effect of mTOR inhibition on podocyte transcription of HIV-mediated downregulation of MnSOD, podocytes treated with the above-mentioned conditions underwent RNA extraction followed by probing for MnSOD. As expected, HIV downregulated podocyte expression of MnSOD; however, rapamycin inhibited this effect (Fig. 10D).

mTOR inhibition attenuates HIV-induced podocyte apoptosis. HP were grown on coverslips and then transduced with either empty vector or HIV in the presence or absence of rapamycin (1 ng/ml). After 48 h, cells were assayed for apoptosis by TUNEL assay (n = 3). HP/HIV showed a greater number of TUNEL+ve cells (P < 0.01) compared with HP. However, the proapoptotic effect of HIV was inhibited (P < 0.05) by rapamycin (HP 1.5 ± 0.6, HP/HIV 8.2 ± 1.1, HP/Rapa 2.1 ± 0.8, HP/HIV/Rapa 3.4 ± 1.2% TUNEL+ve cells/field; n = 6).

p53 is essential for HIV-induced podocyte apoptosis. To determine whether HIV-induced podocyte apoptosis was mediated through p53, HP/HIV were transfected with either p53 siRNA (HP/HIV-p53siRNA) or scrambled siRNA (HP/HIV-sCR). Control, HP/HIV, HP/HIV-p53siRNA, and HP/HIV-sCR were evaluated for percentage of apoptosed cells by TUNEL assay. HP/HIV displayed greater percentage (P < 0.01) of apoptosed cells compared with control (Control 2.8 ± 0.5% vs. HP/HIV 22.8 ± 3.5% apoptosed cells/field; n = 3). On the other hand, HP/HIV lacking p53 displayed decreased percentage (P < 0.01) of apoptosed cells compared with HP/HIV (HP/HIV 22.8 ± 3.5% vs. HP/HIV-p53siRNA 9.0 ± 0.8% apoptosed cells; n = 3). These findings indicate that HIV induces podocyte apoptosis partially through p53 expression.
DISCUSSION

In the present study, renal tissues of HIVAN mice displayed enhanced p53 expression and associated downstream signaling. The latter was associated with enhanced kidney cell oxidative kidney cell damage, apoptosis, glomerulosclerosis, and microcyst formation. On the other hand, mTOR inhibition in HIVAN not only downregulated renal tissue p53 expression and downstream signaling but also provided protection against p53-mediated oxidative kidney cell injury. Additionally, kidney cells in HIVAN mice displayed enhanced phosphorylation of p66ShcA; however, the mTOR inhibition not only downregulated phosphorylation of p66ShcA but also reactivated the redox-sensitive response program in the form of enhanced expression of MnSOD and catalase. In in vitro studies, HIV promoted podocyte apoptosis but mTOR inhibition attenuated this effect of HIV. Similarly, podocytes lacking p53 displayed resistance to apoptosis in the HIV milieu. Since HP/HIV after silencing for mTOR also displayed attenuated expression of...
p53, there seems to be a causal relationship between mTOR and kidney cell p53 expression and associated oxidative kidney cell injury in the HIV milieu.

mTOR signaling contributes toward maintenance of the normal physiological state as well as development of pathological states. For example, it is essential for growth and development but also contributes to the development of diabetes, cardiac hypertrophy, malignancies, neurodegenerative syndromes, and aging (43, 51, 53). In experimental animal models, inhibition of mTOR signaling increases life span (5, 49). Caloric restriction, an intervention that extends life span, has also been implicated to work through the mTOR pathway (41). The mTOR modulation late in life also increases mouse life span. Since oxidative stress is the major player in aging, it is likely that mTOR inhibition might have served as antioxidative therapy. In the present study also, mTOR inhibition mitigated HIV-induced oxidative kidney cell injury.

p53 has been reported to cause inhibition of mTOR activation, whereas mTOR has been reported to activate p53-mediated downstream signaling (8, 9). Interestingly, the mTOR pathway has been reported to contribute to the pathogenesis of diabetic and HIV-associated nephropathy (25, 26). Moreover, the effector molecules of these entities—glucose and HIV—have also been reported to activate both kidney cell mTOR and p53 pathways (23, 35, 36, 46). However, there are no data on cross talk between mTOR and p53 in the development of these diseases. Since both glucose and HIV have been reported to

Fig. 10. mTOR is critical for podocyte p53 expression. A: human podocytes (HP) were transfected with either scrambled (SCR) small interfering RNA (siRNA) or mTOR siRNA. Protein blots of control, HP/SCR-siRNA, and HP/mTOR-siRNA were probed for mTOR. The same blots were reprobed for actin. A representative gel and densitometric analysis are displayed. B: protein blots of control, HIV-transduced HP (HP/HIV), mTOR siRNA-transfected HP/HIV, and scrambled siRNA-transfected HP/HIV were probed for p53 (n = 3). The same blots were reprobed for actin. Cumulative data are shown as bar graphs (n = 3). C: HP and HP/HIV were incubated in medium for 48 h. Total RNA was extracted and probed for p53 (n = 3). Cumulative data are shown as bar graphs. D: HP and HP/HIV were incubated in medium for 48 h. Total RNA was extracted and probed for MnSOD (n = 3). Cumulative data are shown as bar graphs.

Fig. 11. Proposed scheme for the role of mTOR in p53-induced kidney cell injury. HIV enhances reactive oxygen species (ROS) generation in multiple ways, including kidney cell activation of renin-angiotensin system (RAS) and phosphorylation of p66ShcA; the latter phosphorylates Akt. HIV can also phosphorylate Akt through the activation of kinases. Akt-induced phosphorylation of Foxo3A deactivates the redox-sensitive stress program in the form of attenuated generation of SOD and catalase (Cat), leading to accumulation of ROS. Phosphorylation of Akt enhances activation of mTOR, which enhances expression of p53 and associated activation of p66ShcA. ROS-induced DNA damage also activates the p53 pathway, which may lead to the expression of inhibitors of the cell cycle (p21, p27, and GADD45) and proapoptotic molecules such as Fas.
enhance ROS generation by kidney cells, it is likely that ROS-induced kidney cell DNA damage might have stimulated upregulation of the p53 pathway (38). Additionally, both glucose and HIV have been demonstrated to activate the renin-angiotensin system, which has been reported to induce oxidative damage in a variety of cells including kidney cells (3, 12, 38). However, the present study suggests a direct relationship between mTOR and p53 pathways. On that account, inhibition of the mTOR pathway not only downregulated the expression of p53 but also attenuated the downstream signaling and the outcome of the p53 activation.

p53 has been reported to be the modulator of oxidative stress—it downregulates low oxidative stress and exacerbates higher levels of stresses (22). For example, at low levels of oxidative stress, p53 activates antioxidant genes such as sestrin and glutathione peroxidase (1, 6, 16). Additionally, p53 induces the expression of TP53-induced glycolysis and apoptosis regulator, which slows glycolysis and promotes the production of NADPH to decrease ROS levels (50). Moreover, p53 suppresses the expression of phosphoglycerate mutase (PGM), which diminishes mitochondrial oxidative respiration and thus attenuates ROS generation (24). Oxidative DNA damage stimulates p53, which further enhances expression of p21 and p27 to provide time to repair damaged DNA in injured cells (12). On the other hand, at higher levels of oxidative stress p53 induces prooxidative genes including p53-inducible gene 3 (Pig3) and proline oxidase (27, 28). Additionally, it causes apoptosis by enhancing expression of BAX and PUMA (19). These roles of p53 suggest that the severity of oxidative stress determines whether p53 will contribute to or provide protection against cellular injury. In the present study, p53 contributed to HIV-induced oxidative stress by enhancing phosphorylation of p66ShcA and deactivating the redox-sensitive stress response in the form of attenuation of MnSOD and catalase expression in HIVAN mice.

We previously reported (20, 39) that HIV deactivated the redox-sensitive stress response program in kidney cells in vitro and in vivo studies. In these studies HIV stimulated kidney cell phosphorylation of p66ShcA and associated downstream phosphorylation of Foxo3A. The activation of the latter pathway was associated with attenuated generation of MnSOD and catalase (39). It was suggested that attenuated generation of free radical scavengers in the HIV milieu causes accumulation of ROS and associated oxidative stress kidney cell injury in HIVAN. In our present study also, kidney cells displayed enhanced oxidative DNA damage, thus confirming the occurrence of ongoing HIV-induced ROS-mediated kidney cell injury in HIVAN. On the other hand, mTOR inhibition not only enhanced renal tissue expression of MnSOD and catalase in HIVAN mice but also decreased oxidative kidney cell DNA damage. These findings indicate that mTOR inhibition has the potential to reverse HIV-induced deactivation of the redox-sensitive stress response program in HIVAN mice.

Several chemotherapeutic agents have been reported to induce p53-mediated apoptosis through activation of the FasR-FasL pathway (30, 48, 52). These studies suggested that the presence of p53 was critical for expression of FasR in these cells (30). These investigators identified a p53-responsive element within the first intron of the FasR gene and three putative elements within the promoter. The intronic element conferred transcriptional activation by p53 and cooperated with p53-responsive elements in the promoter of the FasR gene. In the present study activation of p53 pathway was associated with renal tissue expression of FasR. The latter seems to be involved in the induction of kidney cell apoptosis in HIVAN mice.

The FasL pathway has been reported to contribute to kidney cell apoptosis in HIVAN (37). In these studies the activation of FasL was attributed to HIV-induced kidney cell NF-kB activation (37). We also demonstrated that HIV enhanced podocyte NF-kB activation (33). Interestingly, mTOR inhibition attenuated HIV-induced kidney cell NF-kB activation (33). In the present study, renal tissues of HIVAN mice also displayed upregulation of FasL. mTOR not only inhibited renal tissue expression of FasL in HIVAN mice but also decreased the number of apoptosed cells. It was likely that this effect of mTOR on inhibition of renal tissue FasL expression was mediated through modulation of NF-kB activation in the HIV milieu.

We have summarized a proposed schematic mechanism delineating the role of mTOR in p53-mediated oxidative kidney cell injury in Fig. 11. Since we have previously reported the role of HIV in the activation of mTOR pathway in kidney cells both in vivo (25) and in vitro (35), we did not emphasize this aspect in the present report. Also, we have previously reported the role of HIV in the activation of p66ShcA pathway and deactivation of the redox-sensitive response program in both podocytes (20) and tubular cells (39).

We conclude that mTOR inhibition provides protection against p53-induced oxidative damage in HIVAN. The present study provides mechanistic insight into the pathogenesis of HIVAN and also provides a basis for the therapeutic strategies for treatment of HIVAN.

REFERENCES


