Molecular Pathogenesis of Genetic and Inherited Diseases

HIV-Associated Nephropathy

Role of Mammalian Target of Rapamycin Pathway

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Both glomerular and tubular lesions are characterized by a proliferative phenotype in HIV-associated nephropathy. We hypothesized that mammalian target of rapamycin (mTOR) contributes to the development of the HIVAN phenotype. Both glomerular and tubular epithelial cells showed enhanced expression of phospho (p)-mTOR in HIV-1 transgenic mice (Tgs). In addition, renal tissues of transgenic mice (RT-Tg) showed enhanced phosphorylation of p70S6 kinase and an associated diminished phosphorylation of eEF2. Moreover, RT-Tgs showed enhanced phosphorylation of 4EBP1 and eIF4B; these findings indicated activation of the mTOR pathway in RT-Tgs. To test our hypothesis, age- and sex-matched control mice and Tgs were administered either saline or rapamycin (an inhibitor of the mTOR pathway) for 4 weeks. Tgs receiving rapamycin not only showed inhibition of the mTOR-associated downstream signaling but also displayed attenuated renal lesions. RT-Tgs showed enhanced expression of hypoxia-inducible factor- α and also displayed increased expression of vascular endothelial growth factor; on the other hand, rapamycin inhibited RT-Tg expression of both hypoxia-inducible factor- α and vascular endothelial growth factor. We conclude that the mTOR pathway contributes to the HIVAN phenotype and that inhibition of the mTOR pathway can be used as a therapeutic strategy to alter the course of HIVAN. (*Am J Pathol* 2010, 177:813–821; DOI: 10.2353/ajpath.2010.100131)

HIV-associated nephropathy (HIVAN) is an important manifestation of AIDS and represents approximately 33%

of biopsy-proven cases of HIV-1-related renal diseases.¹ HIVAN is characterized by a combination of collapsing variant of focal segmental glomerulosclerosis and microcystic dilatation of tubules.² Both of these lesions have a unique proliferative phenotype that is only seen in idiopathic collapsing focal segmental glomerulosclerosis besides HIVAN. However, the exact mechanism contributing to the pathogenesis of the proliferative phenotype of these lesions is not clear. Thus, it may be important to look into the role of the proliferative pathways in the induction of the HIVAN phenotype.

The mammalian target of rapamycin (mTOR) has a central role in the regulation of cell growth.^{3–8} In brief, mTOR is connected upstream to multiple signaling pathways, including growth factors and nutrients to stimulate protein synthesis by phosphorylating key translation regulators such as ribosomal S6 kinase and eukaryotic 4E binding protein (4EBP). An increase in Ser²⁴⁴⁸ phosphorylation (phos) of mTOR enhances Thr³⁸⁹ phos of p70S6 kinase and is indicative of activation of the mTOR pathway; in addition, reduction in eEF2 phos of Thr⁵⁶ is also indicative of p70S6 kinase activation and stimulation of the elongation phase of mRNA translation. The mTOR regulates a wide variety of cellular functions, including translation, transcription, mRNA turnover, protein stability, actin cytoskeletal organization, and autophagy.^{3,4,7,8} The 70-kDa S6 protein kinase and the eukaryotic translation initiation factor (eIF) 4E are pivotal in translation initiation and are required to accelerate the rate of protein synthesis in preparation for cell division. The role of the mTOR pathway in regulation of mRNA translation has been reviewed in depth in renal physiology as well as in renal pathology.^{7,8}

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Rapamycin in complex with FKBP12 interacts with mTOR and inhibits the activity when mTOR is part of mTOR complex 1.⁴ Recently, activation of the mTOR pathway has been shown both in human and experimental animal models of kidney diseases.^{9–13} On that account, rapamycin has been used to attenuate proteinuria associated with allograft nephropathy in human^{10–13} and progression of renal lesions in membranous nephropathy, diabetic nephropathy, and polycystic kidney disease in experimental animal models.^{11–13}

In the present study, we examined the role of mTOR in the development of the HIVAN phenotype in a mouse model of HIVAN. We found that HIVAN mice showed activation of the mTOR pathway both in glomerular and tubular cells. Moreover, inhibition of the mTOR pathway not only attenuated the HIVAN phenotype but also partially inhibited the development of renal lesions.

Materials and Methods

HIV Transgenic Mice

We used age- and sex-matched FVB/N (control) and Tg26 (on an FVB/N background) mice. Breeding pairs of FVB/N mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Breeding pairs to develop Tg26 colonies were a kind gift of Professor Paul E. Klotman, M.D. (Mount Sinai Medical Center, New York, NY). The Tg26 transgenic (Tg) animal has the proviral transgene, pNL4-3:d1443, which encodes all of the HIV-1 genes except *gag* and *pol*, and therefore the mice are noninfectious. These mice developed proteinuria at approximately 24 days of age and progressed into nephrotic syndrome and renal failure.¹⁴ Renal histology revealed focal segmental glomerulosclerosis and microcystic tubular dilatation. Renal tissue showed HIV-1 viral gene expression before the development of renal lesions.¹⁴ In all of our studies, we have used Tg26 mice as HIVAN mice.

Mice were housed in groups of four in a laminar-flow facility (Small Animal Facility, Long Island Jewish Medical Center, New Hyde Park, NY). We are maintaining colonies of these animals in our animal facility. For genotyping of these animals, tail tips were clipped, DNA was isolated, and PCR studies were performed using the following primers for Tg26: HIV forward 5'-ACATGAGCAGTCAGTTCTGCCG-CAGAC-3' and HIV- reverse 3'-CAAGGACTCTGATGCG-CAGGTGTG-5'.

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

Experimental Studies

Six male FVB/N and Tg ($n = 6$) mice aged 4 weeks were sacrificed, and kidneys were isolated as described below. Renal tissues were prepared for renal histology, immunohistochemical, and Western blotting studies.

Rapamycin Studies

Tg26 mice (aged 3 weeks) in groups of six were administered either normal saline (Tg) or rapamycin (5 mg/kg i.p. every other day) (TgR) for 4 weeks. Age- and sex-matched FVB/N mice in groups of six were also administered either normal saline (C) or rapamycin (5 mg/kg i.p. every other day) (CR) for the same duration. These animals served as control for Tgs and TgRs. At the end of the scheduled periods, the animals were anesthetized (by inhalation of isoflurane and oxygen) and sacrificed (by a massive intraperitoneal dose of sodium pentobarbital). After euthanization, blood was collected by a cardiac puncture. Both kidneys were excised; one was processed for histological and immunohistochemical studies, and the other was used for RNA and protein extraction. Three-micrometer sections were prepared and stained with H&E and PAS.

Renal Disease Biomarkers

Five primary phenotypes related to renal disease were characterized: blood pressure, renal histology, proteinuria (urinary protein/creatinine ratio), and biochemical parameters (blood urea nitrogen and serum albumin). Blood pressure (systolic) was measured by the CODA, non invasive blood pressure system (Kent Scientific Corp., Torrington, CT) at 2-week intervals. Proteinuria was measured by an automated analyzer, which quantified the levels as low as 1.0 $\mu\text{g}/\text{ml}$; blood was obtained at the end of the experimental protocol by cardiac puncture (under anesthesia) at the time of sacrifice.

Renal Histology

Renal cortical sections were stained with H&E and PAS. Renal histology was scored for both tubular and glomerular injury. Renal cortical sections were coded and examined under light microscopy. Twenty random fields ($\times 20$)/mouse were examined to score percentage of the involved glomeruli and tubules. A semiquantitative scale (0, no disease; 1, 1–25% of tissue showing abnormalities; 2, 26–50% of tissue affected; 3, >50% of tissue affected) was used for scoring.

Immunohistochemical Staining

The immunohistochemical protocol used in the present study has been described previously.¹⁵ In brief, the sections were deparaffinized, and antigen retrieval was accomplished by microwave heating for 10 minutes at maximum output in 10 mmol/L citrate buffer (pH 6.0). The endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in methanol for 30 minutes at room temperature. Sections were washed in PBS three times and incubated in blocking serum solution according to the primary antibody for 1 hour at room temperature. The primary antibody was applied in different dilutions: phospho-mTOR (1:500, no. 2971, Cell Signaling Technology, Danvers, MA) and vascular endothelial growth factor (VEGF) (mouse monoclonal,

1:1000, no. 7279, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and then incubated overnight at 4°C in a humidifying chamber. Each of the sections was washed three times with PBS and incubated in the appropriate secondary antibody at 1:250 dilutions at room temperature for 1 hour. After sections were washed with PBS three times, they were incubated in ABC reagent (Vector Laboratories, Burlingame, CA) for 30 minutes. Sections were washed again three times in PBS and placed in a Vector Nova RED substrate kit SK-4800 (Vector Laboratories) followed by counterstaining with methyl green. The sections were then dehydrated and mounted with a xylene-free mounting medium (Permount, Fisher Scientific Corporation, Fair Lawn, NJ). In all of the batches of immunostaining, appropriate positive and negative controls were used. All of the immune-stained slides were coded and blindly studied by two investigators using a semiquantitative grading score.

Protein Extraction and Western Blotting

Renal cortical sections from HRenal cortical tissues were mixed with lysis buffer (1× PBS, pH 7.4, 0.1% SDS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1.0 mmol/L sodium orthovanadate, 10 μ l of protease inhibitor cocktail, 100× [Calbiochem, San Diego, CA] per 1 ml of buffer, and 100 μ g/ml phenylmethylsulfonyl fluoride), homogenized with a Dounce homogenizer, and then incubated on ice for 30 minutes. The samples were subjected to centrifugation at 15,000 $\times g$ for 20 minutes at 4°C. The collected supernatant was evaluated for protein concentration as determined by a BCA kit (Pierce, Rockford, IL). The proteins, 20 to 40 μ g/lane, were separated by 10 or 12% SDS-polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane using a Bio-Rad Western blotting apparatus. After transfer, blots were stained with Ponceau S (Sigma-Aldrich, St. Louis, MO) to check for complete protein transfer and equal loading. The blots were blocked with 0.5% bovine serum albumin and 0.1% Tween 20 in 1× PBS for 60 minutes at room temperature and then incubated with the phospho-mTOR (1:500), phospho-p70S6K (1:500, no. 32359, Abcam, Cambridge, MA), phospho-eEF2 (1:500, rabbit polyclonal, Cell Signaling Technology Inc.), phospho-4EBP1 (1:500, rabbit polyclonal, Cell Signaling Technology, Inc.), and phospho-eIF4B (1:500, rabbit polyclonal, Cell Signaling Technology, Inc.) antibodies and VEGF (mouse monoclonal, 1:1000), overnight at 4°C. A horseradish peroxidase-conjugated appropriate secondary antibody was applied for 1 hour at room temperature. The blots were then developed using a chemiluminescence detection kit (ECL, Amersham, Arlington Heights, IL) and exposed to Kodak X-OMAT AR film. Quantitative densitometry was performed on the identified band using a computer-based measurement system.

RT-PCR Analysis

Control and experimental renal tissues were used to quantify mRNA expression of HIF- α . RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA). For cDNA synthesis, 2 μ g of the total RNA was preincubated with 2

nmol of random hexamer (Invitrogen) at 65°C for 5 minutes. Subsequently, 8 μ l of the RT reaction mixture containing cloned avian myeloblastosis virus reverse transcriptase, 0.5 mmol each of the mixed nucleotides, 0.01 mol of dithiothreitol, and 1000 U/ml of RNasin (Invitrogen) was incubated at 42°C for 50 minutes. For a negative control, a reaction mixture without RNA or RT was used. Samples were subsequently incubated at 85°C for 5 minutes to inactivate the RT.

Quantitative PCR was performed in an ABI Prism 7900HT sequence detection system using the following primer sequences: HIF-1 α forward 5'-TCAAGTCAGCAACGTGGAAG-3' and HIF-1 α reverse; 5'-TTACGAGGCTGTGTCGACTG-3'.

SYBR Green was used as the detector and *ROX* as the passive reference gene. Results (means $\pm$ SD) represent three animals as described in the figure legends. The data were analyzed using the comparative C_T method ($\Delta\Delta C_T$ method). Differences in C_T are used to quantify the relative amount of PCR target contained within each well. The data are 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 was applied and a Bonferroni multiple range test was used to calculate a *P* value. Statistical significance was defined as *P* < 0.05.

Results

HIV-1 Transgenic Mice Display HIVAN Phenotype

Renal cortical sections from HIV-transgenic mice showed proliferation of podocytes and collapse of glomerular capillary tufts; moreover, tubules not only showed proliferation but also displayed microcystic dilatation. Representative microphotographs of renal cortical sections from a control and a Tg mouse are shown in Figure 1, A and B.

Renal Cells Show Enhanced Expression of Phospho-mTOR

To evaluate the status of renal cell phosphorylation of mTOR, renal cortical sections of control and Tg mice were immunolabeled for phospho-mTOR. Representative photomicrographs of a control and a Tg mouse are shown in Figure 2A. Control mice showed only minimal labeling for phospho-mTOR in their renal cells, whereas both parietal epithelial and visceral epithelial (podocytes) cells in Tg26 mice showed enhanced labeling for phospho-mTOR. Similarly, in Tg26 mice, tubular cells showed enhanced expression of phospho-mTOR.

Figure 1. HIV-1 transgenic mice display the HIVAN phenotype. Renal cortical sections from control (FVB/N) and Tg26 mice were stained with PAS and evaluated for the development of the HIVAN phenotype. **A:** A representative microphotograph of a renal cortical section from a control mouse shows normal glomeruli and normal tubules. **B:** A representative microphotograph of a renal cortical section from a Tg mouse shows collapsed glomerular capillary tufts (**closed arrows**), sclerosis (**open arrows**), proliferating podocytes (**closed arrowheads**) and a microcyst (**open arrowhead**). Original magnification, $\times 300$.

Renal Tissues Show mTOR Pathway Activation in Tgs

To determine activation of the mTOR pathway, renal cortical tissues from control and Tg mice were prepared for Western blotting and probed for molecules involved in the downstream signaling of the mTOR pathway. Immunoblots of Tgs showed an increase in Ser²⁴⁴⁸ phospho of mTOR and in Thr³⁸⁹ phospho of p70S6 kinase (Figure 2B); both suggest activation of mTOR in the renal cortex. Moreover, reduction in eEF2 Thr⁵⁶ is indicative of an increase in p70S6 kinase

activation and stimulation of the elongation phase of mRNA translation.^{7,8} In addition, enhanced phosphorylation of 4EBP1 and eIF4B indicates initiation of the mRNA translation phase in renal tissue of Tgs. Therefore, renal tissues in Tgs display the stimulation of both initiation and elongation phase of mRNA translation. Ser⁴²² phosphorylation of eIF4B and Ser²⁴⁴⁸ phosphorylation of mTOR are both under p70S6 kinase control.¹⁶ Thus, a positive feedback loop of mTOR stimulation, p70S6 kinase stimulation leading to mTOR activation, seems to exist in HIV kidneys.

Rapamycin Attenuates Progression of HIVAN

To determine the role of mTOR pathway inhibition in the development of the HIVAN phenotype, renal cortical sections of Tgs, and TgRs were evaluated for severity of renal lesions. Representative microphotographs are shown in Figure 3A. Tgs showed a collapsing form of glomerulosclerosis and microcystic dilatation of tubules, whereas TgRs showed attenuated glomerular lesions and minimal dilatation of tubules (Figure 3A). Cumulative data showing percentages of involved glomeruli (sclerosis, grade 0 to 4) and tubules (dilatation, grade 0 to 4) in Tgs and TgRs are shown in Figure 3, B and C, respectively.

Effect of Rapamycin on Proteinuria and Blood Urea Nitrogen Levels

TgRs showed a fourfold decrease in the urinary protein/creatinine ratio compared with Tgs (Figure 4). Similarly, TgRs showed a sevenfold decrease in mean blood urea nitrogen (BUN) levels compared with mean BUN levels in

Figure 2. Activation of mTOR pathway in Tgs. **A:** Renal cortical sections of control and Tg mice were immunolabeled for phospho-mTOR. **Upper panel:** A representative microphotograph of a control mouse shows minimal renal cell labeling for phospho-mTOR. **Middle panel:** A representative microphotograph of a renal cortical section from a Tg mouse shows phospho-mTOR expression by both parietal epithelial (**closed arrows**) and visceral epithelial (**open arrows**) cells. Original magnification, $\times 300$. **Lower panel:** Another representative microphotograph from a Tg mouse shows tubular cell expression of phospho-mTOR (**arrows**). Original magnification, $\times 300$. **B:** mTOR-induced downstream signaling in renal tissue. Renal cortical tissues from two control (C1 and C2) and two Tg (Tg1 and Tg2) mice were prepared for Western blotting and probed for phospho-mTOR, phospho-p70S6K, phospho-eEF2, phospho-4EBP1, phospho-eIF4B, and actin. Phospho-mTOR: immunoblots showing renal tissue expression of phospho-mTOR in control (C1 and C2) and Tg (Tg1 and Tg2) mice. Phospho-p70S6K: immunoblots showing phospho-p70S6K expression by renal tissues from control (C1 and C2) and Tg (Tg1 and Tg2) mice. Phospho-eEF2: immunoblots showing renal tissue expression of phospho-eEF2 in control (C1 and C2) and Tg (Tg1 and Tg2) mice. Phospho-4EBP1: immunoblots showing phospho-4EBP1 by renal tissues from control (C1 and C2) and Tg (Tg1 and Tg2) mice. Phospho-eIF4B: immunoblots showing renal tissue expression of phospho-eIF4B in control (C1 and C2) and Tg (Tg1 and Tg2) mice. Actin: immunoblots showing actin content in renal tissues under above mentioned conditions.

Figure 3. Rapamycin attenuates development of HIVAN. Renal cortical sections of six control (FVBN), Tg, and TgR mice were evaluated for severity of renal lesions. **A:** Representative microphotographs of a control, Tg, and TgR mice show renal histology. Renal cortical section from a control mouse (**left panel**) shows a normal glomerulus and tubules. Renal cortical section from a Tg mouse (**middle panel**) shows collapsed glomerular capillary tufts (**arrowhead**) and microcystic dilatation of tubules (**arrows**). Renal cortical section from a TgR mouse (**right panel**) shows absence of collapsed glomerular capillary tufts (**arrows**) and only mild dilatation of tubules (**arrowheads**). **B** and **C:** Cumulative data showing percentage of the involved glomeruli (**B**, graded 0–4) and tubules (**C**, graded 0–4) are shown in the form of bar diagrams. * $P < 0.001$ compared with respective TgR

Tgs. Interestingly, FVBN mice receiving rapamycin (CR) showed a 2.5-fold increase in proteinuria compared with mice receiving vehicle (C) only (urinary protein/creatinine ratio: C, 0.6 ± 0.2 and CR, 1.5 ± 0.9). These findings indicate that rapamycin is nephrotoxic in control mice; however, in the setting of HIVAN, it clearly attenuates proteinuria in mice.

Rapamycin Down-Regulates Phosphorylation of p70S6 Kinase in Tgs

To determine the effect of rapamycin on mTOR-mediated downstream signaling, renal cortical sections of control, Tgs, and TgRs were immunolabeled for phospho-p70S6K. Immunoblots of renal cortical tissues of Tgs showed enhanced phosphorylation of p70S6 kinase compared with immunoblots of control mice (Figure 5A). However, rapamycin attenuated the phosphorylation of p70S6 kinase in Tgs (Figure 5A). Cumulative data on renal cortical tissue expres-

sion of phosphorylated p70S6 kinase in control and Tg mice are shown in Figure 5B.

Rapamycin Inhibited Renal Tissue Elongation of mRNA Translation Phase in Tgs

To determine whether rapamycin-induced down-regulation of the mTOR pathway was also associated with enhanced phosphorylation of Thr⁵⁶ of eEF2, we immunolabeled renal tissues of control, Tg, and TgR mice for phospho-eEF2. Renal tissues from Tgs showed attenuation of phos of Thr⁵⁶ of eEF2 (Figure 5C), which was

Figure 4. Effect of rapamycin on proteinuria and BUN levels. At the end of the experimental protocol, urinary protein: creatinine ratio and BUN levels were measured in control and Tgs receiving either normal saline or rapamycin. TgRs show fourfold decrease in urinary protein: creatinine ratio when compared with Tgs. TgR also show sevenfold decrease in mean BUN levels when compared with mean BUN levels of Tgs. * $P < 0.01$ compared with Tg; ** $P < 0.01$ compared with Tg.

Figure 5. Rapamycin attenuates renal tissue phosphorylation of p70S6 kinase and eEF2. **A:** Renal tissues from two control, Tg, and TgR mice were prepared for Western blotting and then probed for phospho-p70S6K and actin. The **upper panel** shows renal tissue expression of phospho-p70S6K by control, Tg, and TgR mice. The **lower panel** shows renal tissue actin content under similar conditions. **B:** Cumulative data ($n = 3$) on renal cortical tissue expression of phospho-p70S6 kinase under the above mentioned conditions. * $P < 0.01$ compared with control; ** $P < 0.05$ compared with Tg. **C:** Renal tissues from three control, Tg, and TgR mice were prepared for Western blotting and subsequently probed for phospho-eEF2 (Thr⁵⁶) and actin. The **upper panel** shows renal tissue expression of phospho-eEF2 (Thr⁵⁶) of two control (C1 and C2), Tg (Tg1 and Tg2), and TgR (TgR1 and TgR2) mice. The **lower panel** shows renal tissue actin content under similar conditions. **D:** Cumulative data on renal cortical tissue expression of phospho-eEF2 (Thr⁵⁶) under the above-mentioned conditions. * $P < 0.01$ compared with control; ** $P < 0.05$ compared with Tg.

Figure 6. Rapamycin inhibits renal tissue expression of phospho-4EBP1. Renal tissues from two control, Tg, and TgR mice were prepared for Western blotting and subsequently were probed for phospho-4EBP1. The **upper panel** shows renal tissue expression of phospho-4EBP1 by control, Tg, and TgR mice. The **lower panel** shows renal tissue actin content under similar conditions.

indicative of the activation of p70S6 kinase and elongation of the mRNA translation phase.¹⁷ On the other hand, renal tissues from TgRs showed enhanced phosphorylation of Thr⁵⁶ of eEF2, thus indicating inhibition of the elongation phase of mRNA translation. Cumulative data on immunoblot analysis of three animals are shown in Figure 5D.

Rapamycin Inhibits Renal Tissue Initiation of mRNA Translation Phase

To determine the effect of rapamycin on the modulation of mTOR-induced downstream signaling, renal cortical tissues of control, Tg, and TgR mice were immunolabeled for phospho-4EBP1 and phospho-eIF4B. Tgs showed enhanced renal tissue phosphorylation of both 4EBP1 (Figure 6) and eIF4B (Figure 7), thus confirming the activation of the mTOR pathway.¹⁷ On the other hand, TgRs showed attenuated phosphorylation of 4EBP1 (Figure 6) and of eIF4B (Figure 7). These findings indicated that rapamycin inhibited the initiation of mRNA translation phase in Tgs.

Rapamycin Attenuated Renal Cell VEGF Expression in Tgs

A proliferative podocyte phenotype in HIVAN has been attributed to the increased podocyte VEGF expression.¹⁸ Both glomerular and tubular cells in Tgs showed enhanced expression of VEGF (Figure 8A). However, rapamycin inhibited renal cell expression of VEGF in Tgs. Immunoblots from these transgenic mice also showed enhanced renal tissue expression of VEGF (Figure 8B); however, rapamycin attenuated renal tissue VEGF expression. Cumulative densitometric data on renal tissue VEGF expression in control, Tg, and TgR mice are shown in Figure 8C.

Figure 7. Rapamycin inhibits renal tissue expression of phospho-eIF4B. Renal tissues from three control, Tg, and TgR mice were prepared for Western blotting and subsequently probed for phospho-eIF4B. The **upper panel** shows renal tissue expression of phospho-eIF4B by control, Tg, and TgR mice. The **lower panel** shows renal tissue actin content under similar conditions.

Rapamycin Inhibited Renal Tissue HIF1- α Expression in Tgs

Because HIF1- α is the transcription factor for VEGF,¹⁹ we suspected that rapamycin might be modulating renal cell VEGF expression by altering renal cell HIF1- α expression. Moreover, mTOR activation has been demonstrated to be associated with enhanced expression of HIF1- α .¹⁹ As shown in Figure 9, renal tissues of Tgs showed enhanced expression of HIF- α mRNA. These findings are consistent with the observations made by other investigators.²⁰ However, rapamycin-receiving mice showed attenuated renal tissue expression of HIF1- α mRNA. These findings indicate that rapamycin may be modulating renal cell VEGF expression by altering the renal tissue expression of HIF1- α .

Discussion

In the present study, glomerular and tubular epithelial cells of control mice showed minimal phosphorylation of mTOR, whereas both parietal and visceral (podocytes) epithelial cells in Tgs showed enhanced phosphorylation of mTOR. Similarly, in Tgs, tubular cells showed enhanced expression of phospho-mTOR. Renal immunoblots of Tgs showed an increase in Ser²⁴⁴⁸ phos of mTOR and in Thr³⁸⁹ phos of p70S6 kinase; these findings indicate the activation of the mTOR pathway in the renal cortices of Tgs. Similarly, a reduction in eEF2 phos of Thr⁵⁶ also indicated the activation of p70S6 kinase and stimulation of the elongation phase of mRNA translation in Tgs. In addition, renal tissues in Tgs showed enhanced phosphorylation of 4EBP1 and eIF4B, thus indicating stimulation of the initiation of mRNA translation phase; these findings further indicated the activation of mTOR pathway in renal tissues of Tgs. On the other hand, immunoblots from renal cortices of TgRs showed attenuated Thr³⁸⁹ phos of p70S6K, thus suggesting inhibition of mTOR pathway. Moreover, renal cortical immunoblots of TgRs showed an increase in eEF2 phos of Thr⁵⁶ (compared with Tgs), which is indicative of inhibition of p70S6 kinase and associated reduction of the elongation phase of mRNA translation. TgRs not only displayed an attenuated HIVAN phenotype but also showed less advanced renal lesions and a reduction in proteinuria and blood urea nitrogen levels.

HIVAN is an exclusive disorder of patients of African descent.¹ Its manifestation depends on specific genetic, environmental, and host factors.^{21–23} Because peripheral blood mononuclear cells from HIV-1-infected patients exhibited an increase in mTOR expression,²⁴ it seems that the mTOR pathway may also be contributing to the dysregulated immune system in these patients. The recent identification of *MYH9* as a susceptible allele is a key step forward in our understanding of the pathogenesis of focal glomerulosclerosis in people of African descent.²⁵ Therefore, to develop a HIVAN phenotype in an HIV-1-infected person, a genetic susceptibility gene such as *MYH9* may be required.

Figure 8. Rapamycin attenuates renal cell VEGF expression in Tgs. **A:** Renal cortical sections of control, Tg, and TgR mice were immunolabeled for VEGF. A representative microphotograph of a renal cortical section from a control mouse shows mild VEGF expression by both glomerular (closed arrows) and tubular (open arrows) cells. A representative microphotograph of a renal cortical section from a Tg mouse (middle panel) shows enhanced VEGF expression both by glomerular (closed arrows) and tubular (open arrows) cells. A representative microphotograph of a renal cortical section from a TgR mouse shows only mild VEGF expression by tubular cells (open arrows). **B:** Renal tissues harvested from control, Tg, and TgR mice were prepared for Western blotting and then probed for VEGF and actin. The upper panel shows renal tissue expression of VEGF by control (C1, C2, and C3), Tg (Tg1, Tg2, and Tg3), and TgR (TgR1, TgR2, and TgR3) mice. The lower panel shows actin content under similar conditions. **C:** Cumulative densitometry data on renal tissue VEGF expression ($n = 3$). * $P < 0.05$ compared with control; ** $P < 0.01$ compared with Tg.

Activation of mTOR has emerged as a regulatory mechanism that is conserved from yeast to mammals in the control of protein biosynthesis and cell size.³ mTOR is a large serine/threonine protein kinase that is found in two distinct multiprotein complexes: mTOR complex 1 (mTORC1, containing raptor and mLST8), which has been implicated in translational regulation,³ and mTORC2 (containing mLST8, mSin1, and rictor).⁴ In an indirect way mTORC2 can also regulate translation. It phosphorylates Akt Ser⁴⁷³, which activates Akt. Prolonged incubation with rapamycin can also inhibit mTORC2 in addition to mTORC1.⁵ Rapamycin in complex with FKBP12 interacts with mTOR and inhibits the activity when mTOR is part of mTORC1.⁴ mTORC1 enhances cell proliferation by stimulating the translation of mRNA and synthesis of proteins necessary for an increase in cell size and progression through the cell cycle.⁶ Translation of mRNA comprises initiation, elongation, and termination phases.^{7,8} The initiation phase mediates the association of ribosomal subunits with mRNA, whereas amino acids are sequentially added to the nascent peptide chain during the elongation phase in accordance with mRNA-determined codon sequences.^{3,4,7}

Tao et al²⁶ first suggested the role of the mTOR pathway in the pathogenesis of adult polycystic kidney disease. These investigators demonstrated that rapamycin slowed cyst formation in the Han:SPRD rat model of

polycystic kidney disease. Shillingford et al¹¹ reported that rapamycin not only slowed cyst enlargement in a murine model of polycystic kidney disease but also slowed the increase in size of native kidneys of humans with autosomal dominant polycystic kidney disease who had received a renal transplant. Recently, Wu et al²⁷ reported that mTOR inhibition effectively controls cyst growth but leads to severe parenchymal and glomerular hypertrophy in rat polycystic kidney disease. Currently, there are three clinical trials underway to look into the therapeutic role of mTOR inhibition in human autosomal dominant polycystic kidney disease.²⁸

In a diabetic model, rapamycin has been reported to attenuate the activation of the mTOR pathway.²⁹ Inhibition of the mTOR pathway was associated not only with amelioration of diabetic glomerular phenotype but also with a reduction in albuminuria.¹² In addition, rapamycin inhibited the influx of inflammatory cells in the interstitium.¹² In a rat model of membranous nephropathy, rapamycin inhibited glomerular hypertrophy and renal expression of proinflammatory and profibrotic cytokines and slowed the rate of progression of tubulointerstitial inflammation and fibrosis.³⁰ Similarly, a slowed rate of progression of renal lesions, tubulointerstitial inflammation, and fibrosis has been reported in Thy-1 antibody-mediated chronic glomerulonephritis and renal ablation models.^{13,31}

mTOR signaling has been considered to be linked to the evolution of kidney hypertrophy due to compensatory growth after the loss of a certain number of nephrons in various models of chronic kidney diseases.³² In a uninephrectomy model, the remaining kidney showed enhanced phosphorylation of p70S6K and 4EBP1.³² Interestingly, rapamycin not only blocked unilateral nephrectomy-induced phosphorylation of p70S6K and 4EBP1 but also inhibited renal hypertrophy.³² Similarly, S6 kinase knockout mice did not develop renal hypertrophy in either the uninephrectomy or diabetic model.³³ Wu et al³⁴ used this strategy to slow down the progression of renal interstitial fibrosis in a mouse unilateral ureteral obstruction model. These investigators demonstrated that rapamycin attenuated the enlargement of the obstructed kidney by reducing tubular

Figure 9. Rapamycin (Rapa) inhibited renal tissue HIF1- α expression. Total RNA was extracted from renal tissues of control, Tg, and TgR mice, and mRNA expression for HIF1- α was assayed by real-time PCR. * $P < 0.05$ compared with other variables.

dilatation and interstitial volume.³⁴ In addition, rapamycin decreased the infiltration of inflammatory cells and inhibited renal transforming growth factor- β 1 expression. The beneficial effect of rapamycin in this model was attributed to the inhibition of the mTOR pathway blocking the increased protein synthesis and cell cycle progression in the obstructed kidney. In the present study, rapamycin attenuated tubular dilatation in TgRs. Because dilated tubules are composed of an increased number of tubular cells as well as elongated basement membrane, we speculate that rapamycin prevented dilatation of tubules in Tg26 by inhibition of both protein synthesis and cell cycle progression in tubular cells.

Several reports indicate that VEGF is required for the maintenance of normal podocyte function—glomerular permselectivity.³⁵ Because VEGF is required for glomerular and tubular hypertrophy and proliferation, its deficiency is associated with the development of glomerulosclerosis in the remnant kidney model.³⁵ Therefore, inhibition of VEGF activity not only induced massive proteinuria but also was associated with the progression of glomerulosclerosis and interstitial fibrosis in a mouse model of crescentic glomerulonephritis.³⁶ On the other hand, podocyte-specific constitutive overexpression of VEGF led to collapse of glomerular capillary tufts.³⁷ These reports indicate that both an excess of and a reduction in VEGF are associated with glomerular pathology.

The activation of the mTOR pathway promotes the expression of HIF- α , a transcription factor for VEGF.²⁰ VEGF has also been demonstrated to enhance podocyte and tubular cell proliferation.^{38,39} Increased vascular endothelial growth factor expression by podocytes has been reported to contribute to the proliferative phenotype in Tg26 mice.¹⁸ In this study, HIV-1 Nef was implicated for the enhanced podocyte VEGF expression.¹⁸ Because mTOR-mediated downstream signaling has been demonstrated to contribute to the transcription of VEGF, we asked whether rapamycin also modulated renal tissue VEGF expression in Tgs. In the present study, renal cortices showed enhanced VEGF expression in Tg mice, whereas renal cortices of TgRs displayed attenuated expression of VEGF. Thus, it appears that the mTOR pathway also contributed to the proliferative phenotype through enhanced renal tissue expression of VEGF.

Use of rapamycin has been reported to induce hyperlipidemia as well as proteinuria in both human and animal models of renal diseases.^{40,41} It has also been demonstrated that mTOR inhibition slows down renal cell injury repair in a model of acute kidney injury.⁴² In the present study, control mice receiving rapamycin showed enhanced proteinuria compared with control mice receiving only vehicle. On the other hand, TgRs showed decreased proteinuria compared with Tgs. These findings are consistent with the observations made by several investigators in other kidney disease models.^{11–13}

We conclude that mTOR pathway activation is contributing to both the proliferative phenotype and the development of HIVAN.

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