Role of the Akt/FoxO3a Pathway in TGF-β1–Mediated Mesangial Cell Dysfunction: A Novel Mechanism Related to Diabetic Kidney Disease

Mitsuo Kato,* Hang Yuan,* Zhong-Gao Xu,‡ Linda Lanting,* Shu-Lian Li,* Mei Wang,* Mickey C.-T. Hu,‡ Marpadga A. Reddy,* and Rama Natarajan*

*Department of Diabetes, Beckman Research Institute of the City of Hope, Duarte, California; ‡Department of Nephrology, 2nd Hospital of Jilin University, Changchun, China; and Departments of Molecular and Cellular Oncology, University of Texas, M.D. Anderson Cancer Center, Houston, Texas

Diabetic nephropathy (DN) is characterized by mesangial cell (MC) expansion and accumulation of extracellular matrix proteins. TGF-β is increased in MC under diabetic conditions and in DN and activates key signaling pathways, including the phosphoinositide-3-kinase/Akt (PI3K/Akt) pathway. FoxO transcription factors play roles in cell survival and oxidative stress and are negatively regulated by Akt-mediated phosphorylation. We tested whether phosphorylation-mediated inactivation of FoxO3α by TGF-β can mediate MC survival and oxidative stress. TGF-β treatment significantly increased levels of p-Akt (activation) and p-FoxO3α (inactivation) in cultured MC. This FoxO3α inactivation was accompanied by significant decreases in the expression of two key FoxO3α target genes, the proapoptotic Bim and antioxidant manganese superoxide dismutase in MC. TGF-β treatment triggered the nuclear exclusion of FoxO3α, significantly inhibited FoxO3α transcriptional activity, and markedly protected MC from apoptosis. A PI3K inhibitor blocked these TGF-β effects. It is interesting that p-Akt and p-FoxO3A levels also were increased in renal cortical tissues from rats and mice at 2 wk after the induction of diabetes by streptozotocin, thus demonstrating in vivo significance. In summary, TGF-β and diabetes can increase FoxO3α phosphorylation and transcriptional inactivation via PI3K/Akt. These new results suggest that Akt/FoxO pathway regulation may be a novel mechanism by which TGF-β can induce unopposed MC survival and oxidant stress in early DN, thereby accelerating renal disease.


Diabetes is a major factor that leads to ESRD and accounts for almost half of all patients who begin dialysis. Numerous factors contribute to the pathogenesis and progression of diabetic nephropathy (DN). Histologically, DN is characterized by glomerular basement membrane thickening and mesangial expansion. This matrix accumulation is due to coordinate alterations in extracellular matrix (ECM) proteins such as types I and IV collagen, laminin, and fibronectin (1–3); ECM regulatory enzymes such as matrix metalloproteinases and tissue inhibitors of matrix metalloproteinases (4,5); and growth factors such as PDGF, TGF-β1, and angiotensin II (6–9).

Factors that are relevant to the pathogenesis of DN can increase TGF-β expression in MC in vitro (7,10–12) and in vivo (7,13,14). Therefore, TGF-β has been studied as a major target for DN treatment. TGF-β is a profibrotic agent with several effects in renal cells, including the production of ECM proteins, type I and II collagens, laminin, heparin sulfate proteoglycan, fibronectin, and also plasminogen activator inhibitor-1 (7,13–17). Improvement in glomerular filtration rates was induced by an anti–TGF-β antibody in experimental DN (18). However, the subtle upstream signal transduction mechanisms by which TGF-β regulates MC dysfunction are not fully clear. Importantly, whereas most of the pathologic effects of TGF-β in the kidney and DN have been attributed to its profibrotic effects, other potential mechanisms have been less well explored.

Interaction of TGF-β with its receptors induces the phosphorylation and nuclear translocation of the receptor-regulated Smad2/3 transcription factors (19–21). MC express Smad 2,3, and Smad 6,7 (inhibitory Smads), which have been shown to regulate TGF-β–induced gene expression (16,17,22,23). Apart from the Smad pathway, the matrix-inducing actions of TGF-β also may be mediated via activation of mitogen-activated protein kinases (MAPK) such as p38 and extracellular signal–regulated kinases (12,24,25).

Phosphoinositide-3-kinase (PI3K) plays a crucial role in cell growth and cell survival (26). PI3K enzyme acts on membrane PI to generate the second messenger lipid PI-3,4,5-triphosphate. PI-3,4,5-triphosphate recruits phosphatidylinositol-dependent kinase 1 and Akt kinase to the membrane, then phosphatidylinositol-dependent kinase 1 phosphorylates and activates Akt. Activated Akt phosphorylates several downstream proteins,
including GSK3-β, Forkhead (FoxO) transcription factors, and tuberous sclerosis 1 and 2 to control cell growth, cell survival, and protein synthesis (26).

TGF-β activates the PI3K/Akt pathway in various cells (27,28). However, the mechanism of TGF-β-mediated PI3K activation and downstream targets in MC are not completely clear. In MC, TGF-β–induced activation of PI3K/Akt and phosphorylation of Smad3 were linked with upregulation of collagen type I a2 gene, suggesting that TGF-β–induced ECM accumulation in MC may be regulated through the PI3K/Akt pathway (27). PI3K/Akt pathway activation also was reported in TGF-β–mediated epithelial to mesenchymal transition and migration (28). Physical interaction between TGF-β type I or II receptors and PI3K was detected in human airway smooth muscle cells and COS7 cells that expressed TGF-β receptors and p85 subunit of PI3K (29,30). Therefore, TGF-β seems to activate the PI3K/Akt pathway in several cell types via association of TGF-β receptors and PI3K.

FoxO3a is a member of FoxO subfamily of Forkhead transcription factors, which are orthologs of Caenorhabditis elegans Daf-16 that are known to regulate longevity (31). Akt can phosphorylate and inactivate three FoxO proteins, FoxO1/FKHR, FoxO3a/FKHRL1, and FoxO4/AFX (32). In the presence of survival factors, Akt phosphorylates FoxO3a, leading to association with 14-3-3 proteins, nuclear exclusion, and retention of FoxO3a in the cytoplasm. Conversely, withdrawal of survival factors leads to FoxO3a dephosphorylation, nuclear translocation, and activation of FoxO target genes (32). FoxO3a regulates cell death by inducing the proapoptotic gene, Bim, in cancer cells, neurons, and endothelial progenitor cells (33–36). FoxO3a also protects cells from oxidant stress by upregulating the antioxidant gene manganese superoxide dismutase (MnSOD) (37). Cross-talk between TGF-β and FoxO3a signaling has been shown in the control of neuroepithelial and glioblastoma cell proliferation (38). PDGF can induce FoxO3a phosphorylation in MC via PI3K/Akt (39), leading to inhibition of FoxO3a phosphorylation.

Although TGF-β activates the PI3K/Akt pathway also in MC

Figure 1. TGF-β induces Akt and FoxO3a phosphorylation in cultured mesangial cells in a phosphoinositide-3-kinase (PI3K)-dependent manner. (A) Immunoblotting analysis of rat mesangial cells (MC) after treatment with TGF-β (10 ng/ml for 5 min to 24 h). TGF-β increased phosphorylation of Akt, FoxO3a at Ser 253, and GSK3β. No change in phosphorylation at Ser644 of FoxO3a was observed. (B) Pretreatment with a PI3K inhibitor, 20 μM of LY294002 (LY) for 1 h blocks, TGF-β–induced FoxO3a phosphorylation. Results shown are representative of three to five experiments.

Figure 2. TGF-β decreases the expression of a key FoxO target gene, the proapoptotic Bim, in MC. (A) Reverse transcriptase–PCR (RT-PCR) analysis of isoforms of the proapoptotic gene Bim in rat MC. Three splicing isoforms, BimEL (extra long), BimL (long), and BimS (short), were detected. TGF-β treatment (6 and 24 h) decreased their expression. 18S was used as an internal control. (B) Relative expression of BimEL. Three independent cultures were studied at each time point. After 6 h of TGF-β treatment, a significant decrease in the expression of BimEL was observed (*P < 0.01). (C) RT-PCR analysis of Bim isoforms in mouse MC. TGF-β inhibits serum depletion–induced increase in expression of Bim isoforms in mouse MC. NT, no treatment; SD, serum depletion.
and inhibition of PI3K or a dominant negative Akt construct could inhibit TGF-β–induced fibronectin expression (40), it is not known whether TGF-β regulates FoxO transcription factors and their downstream targets in renal cells. In this study, we observed for the first time that TGF-β induces phosphorylation-induced transcriptional inactivation of FoxO3a in MC via the PI3K pathway and that this leads to decreased expression of its downstream targets, MnSOD and Bim. Furthermore, we noted increases in FoxO3a phosphorylation in renal cortical tissues of diabetic animals. Our results suggest that TGF-β–induced inactivation of FoxO3a may be a key additional novel mechanism in the pathogenesis of DN.

Materials and Methods

Cell Culture

Rat and mouse MC were obtained and cultured as described previously (12) in RPMI-1640 supplemented with 10% FBS. Recombinant human TGF-β1 was from R&D System (Minneapolis, MN). PI3K inhibitor LY294002 (LY) was from Calbiochem (La Jolla, CA).

Animals

All animal studies were conducted under a protocol approved by the Institutional Research Animal Care Committee. Male Sprague-Dawley rats received a single injection of 65 mg/kg streptozotocin (STZ) intraperitoneally. C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME) received an injection of 50 mg/kg STZ intraperitoneally on 5 consecutive days as described previously (41). Blood glucose was measured to confirm the development of diabetes (fasting glucose >300 mg/dl). Rats and mice that received an injection of diluent buffer alone served as control. All animals were killed 2 or 16 wk after the onset of diabetes. Sieved glomeruli from rat kidneys and cortical tissues from mouse kidneys were removed as described (41) and stored at −70°C for further study.

Western Blot Analysis

Cells and kidney tissues were lysed in SDS sample buffer (2% SDS, 10 mM Tris-HCl [pH 6.8], and 10% glycerol). Proteins were separated by SDS-PAGE, transferred to membranes, and detected with appropriate antibodies as described previously (42). Antibodies against total FoxO3a, phospho253Ser-FoxO3a, Akt, GSK-3β, and actin were from Cell Signaling (Beverly, MA). Antibody against phospho644Ser-FoxO3a was described previously (43). Blots were scanned using GS-800 densitometer, and bands were quantified with Quantity One software (Bio-Rad Laboratory, Hercules, CA).

Reverse Transcriptase–PCR

Total RNA was extracted using STAT-60 according to the manufacturer’s instructions (TEL-TEST Friendswood, TX). A total of 0.5 or 1 μg of total RNA was subjected to reverse transcription using GeneAmp RNA PCR Kit (Applied Biosystems, Foster City, CA). cDNA were PCR-amplified using appropriate primers. Primer sequences used were as follows: Bim, forward GCGAAGCAACCTTCTGATGTA and reverse CAGTGCCTTCTCCAGACCAG; and MnSOD, forward GACCTGCTCTACGACTATGG and reverse GACCTTGCTCCTTATTGAAGC. 18S RNA was used as an internal control in these relative reverse transcriptase–PCR. 18S primers were from Ambion (Austin, TX). PCR products were separated on agarose gels and stained with ethidium bromide. Bands were quantified with Quantity One software and normalized to 18S.

Real-Time PCR

Real-time PCR was performed using SYBR Green PCR Master Mix and 7300 Realtime PCR System (Applied Biosystems), according to the manufacturer’s protocols.

Immunohistochemistry

Paraffin sections of rat and mouse kidneys were mounted onto positive charged slides, deparaffinized, washed with water, blocked with Dako protein block (Dako, Carpinteria, CA), and incubated with ×100 dilution of phospho253Ser-FoxO3a antibody overnight. Slides were washed with Dako wash, treated with hydrogen peroxide for 5 min, washed with PBS, incubated with anti-rabbit and mouse secondary antibody conjugated with a peroxidase polymer (Dako), washed, and incubated with 3,3’-diaminobenzidine for 5 min. Slides were counterstained in 50% Mayer’s hematoxylin for 1 min.

Nuclear Translocation of FoxO3a-Green Fluorescence Protein

Rat MC were plated in 12-well dishes (100,000/well) and transfected with pFoxO3a–green fluorescence protein (GFP) fusion construct (43)
using FuGENE 6 transfection reagent (Roche, Indianapolis, IN). Cells were serum-depleted and treated with TGF-β or 10% FBS. Treated cells were fixed in 3% paraformaldehyde, and cellular localization of fusion protein was observed by fluorescence microscopy (Olympus, Tokyo, Japan). For detection of the nuclei, cells were stained with 0.2% Hoechst 33342 (Molecular Probes, Eugene, OR). Nuclear condensation also was detected by Hoechst staining.

**Luciferase Assays**

Rat or mouse MC were transfected with pFRE-luc (43) using FuGENE 6 Transfection Reagent and treated with TGF-β/H9252 with or without PI3K inhibitor LY. After 24 h, luciferase activities were measured using Luciferase Assay System (Promega, Madison, WI) and TD-20/20 luminometer (Turner Designs, Sunnyvale, CA), according to the manufacturer’s instructions. pCI-neo-HA-FoxO3a (wild-type FoxO3a expression vector) and pECE-FoxO3a-(A)3 (Akt site mutant; T32A, S253A, and S315A) also used for reporter experiments were described previously (43).

**DNA Ladder Formation**

Genomic DNA was extracted from cultured cells by standard methods (44). For the detection of genomic DNA fragmentation, 1 μg of purified DNA was run on 2% agarose gels, stained with 0.5 μg/ml ethidium bromide, and visualized under ultraviolet light.

**Results**

**Phosphorylation of FoxO3a in Rat MC by TGF-β**

We first examined the effects of TGF-β on the phosphorylation of FoxO3a, Akt, and GSK3b by Western blot analyses using phospho-specific antibodies. Figure 1 shows that treatment of rat MC with TGF-β (10 ng/ml) increased the phosphorylation of Akt, FoxO3a (at Ser 253, Akt phosphorylation site), and GSK3b (a known target of Akt; Figure 1A). Increases in p-AKT and p-FoxO3a levels were observed as early as 5 min after TGF-β treatment. Evidence shows that FoxO3a also can be phosphorylated by IκB-kinase (IKK) at Ser644 (Figure 1A). Thus, TGF-β seems to phosphorylate FoxO3a via Akt and not IKK.

Akt is a downstream effector of PI3K (26,45). Because Akt is a major kinase that is known to phosphorylate FoxO3a, we tested whether inhibition of PI3K can reduce FoxO3a phosphorylation. As expected, TGF-β–induced FoxO3a phosphorylation was blocked in rat MC that were pretreated for 1 h with the PI3K inhibitor LY (Figure 1B).

**FoxO3a Downstream Target Genes Are Inhibited by TGF-β Treatment**

Because FoxO3a transcriptional activity is lost upon phosphorylation, we next examined whether TGF-β can downregulate the expression of key FoxO target genes, namely Bim (a proapoptotic gene) and MnSOD (an antioxidant gene) by reverse transcriptase–PCR of total RNA that was isolated from TGF-β–stimulated MC. Results showed that three isoforms of Bim (BimEL [extra long], BimL [long], and BimS [short]) were detected in the MC, and the mRNA levels of all three isoforms were decreased by TGF-β treatment (Figure 2A). The reduction in BimEL mRNA was significant at 6 and 24 h (Figure 2B). We next noted that these three Bim isoforms also were present in mouse MC (Figure 2C) under normal conditions (no treatment). This was increased by serum depletion, and TGF-β treatment (6 and 24 h) clearly decreased the expression of three isoforms (Figure 2C).

**Figure 4. TGF-β leads to nuclear exclusion of FoxO3a in mesangial cells.** Rat MC were transfected with a plasmid that express FoxO3a–green fluorescence protein (GFP) fusion protein, serum-depleted, and treated with TGF-β. Under normal conditions (serum +), FoxO3a was localized in cytoplasm (A). Upon serum depletion, FoxO3a accumulated in nuclei (B) and TGF-β treatment induced nuclear exclusion (C). Cells also were stained with Hoechst dye to visualize the nuclei (D through E).
alter intracellular localization of FoxO3a, rat MC were transfection with a plasmid that expresses FoxO3a-GFP fusion protein (43). Transfected cells were serum-depleted, treated with TGF-β, and fluorescence monitored to examine the nuclear localization of FoxO-GFP protein. Under normal growth conditions in the presence of serum (control), FoxO3a was localized primarily in the cytoplasm (Figure 4A). Serum depletion led to the nuclear accumulation of FoxO3a (Figure 4B), and this was reversed by TGF-β treatment, which led to nuclear exclusion (Figure 4C). Nuclei were identified by Hoechst staining (Figure 4, D through F). These results indicate that, in the presence of serum, FoxO3a is phosphorylated, remains inactive, and is localized in cytoplasm. Serum depletion induces FoxO3a dephosphorylation and leads to its translocation into the nucleus, where it can induce the expression of targets Bim

and MnSOD, as shown previously (Figures 2 and 3). TGF-β treatment can reverse this effect by inducing FoxO3a phosphorylation and its nuclear exclusion, resulting in inhibition of Bim and MnSOD expression as a result of FoxO3a inactivation.

Inhibition of Transcription from Forkhead Response Element by TGF-β

Because we observed a decrease in the expression of FoxO3a target genes, as well as nuclear exclusion of FoxO3a by TGF-β treatment, we next examined whether TGF-β can reduce the transcriptional activity of FoxO. Rat and mouse MC were transfected with the reporter plasmid Forkhead responsive element (FRE)-Luc that contained luciferase reporter gene driven by an FRE (43) and determined luciferase activity after treatment with or without TGF-β. Figure 5 shows that TGF-β treatment significantly decreased the luciferase activity in both rat (Figure 5A) and mouse (Figure 5B) MC. Pretreatment with the PI3K inhibitor restored the transcriptional activity (TGF-β LY) to nearly the levels that were seen in control cells (no treatment) in both rat and mouse MC (Figure 5, A and B). To
test the direct involvement of FoxO3a, pCI-neo-FoxO3a-(A)₃ that contained mutations in three Akt phosphorylation sites (T32A, S253A, and S315A) was co-transfected with pFRE-luc (Figure 5C). Because this mutant cannot be phosphorylated by Akt, it acts as constitutively active FoxO3a and depicts much greater basal FRE-luc activity (almost seven-fold) than does wild-type FoxO3a (Figure 5C). Furthermore, as expected, unlike with WT-Foxo3a, FRE-luc reporter activity was not decreased by TGF-β in cells that were transfected with FoxO3a-(A)₃ (Figure 5C). These results further support the role of the PI3K/Akt pathway in mediating TGF-β-induced FoxO3 phosphorylation and transcriptional inactivation.

**TGF-β Supports MC Survival**

Because we noted a significant decrease in the expression of Bim, a key proapoptotic gene, we hypothesized that TGF-β (or diabetic conditions) can promote MC survival. We therefore tested whether TGF-β can reverse serum depletion–induced apoptosis in MC. We first evaluated the appearance of condensed nuclei, a key marker of cellular apoptosis. As expected, serum depletion of mouse MC increased the number of condensed nuclei relative to control (Figure 6), and this was reversed significantly by TGF-β (Figure 6, Table 1). We then examined DNA ladder formation, another index of apoptosis. Serum-depleted mouse MC showed clearly increased DNA ladder formation, and TGF-β treatment could reverse this (Figure 7). These results support our hypothesis that TGF-β protects MC from serum depletion–induced apoptosis, and this could be via FoxO3a inactivation.

**Phosphorylation of FoxO3a in Kidneys of Diabetic Rats and Mice**

To examine further the potential relationship to DN and in vivo relevance, we next examined whether FoxO3a phosphorylation levels were altered in glomeruli and cortical tissues from diabetic rats and mice, respectively. Figure 8A shows results of immunoblotting with antibodies to p-FoxO3a and p-Akt in glomeruli that were obtained from control and STZ-induced diabetic rats (2 wk after onset of diabetes). Quantification of data shows that levels of p-Akt (Figure 8B) and pFoxO3a (Figure 8C) were increased significantly (eight-fold and four-fold, respectively) in glomeruli of diabetic rats relative to those from controls. However, there was no difference in FoxO1 phosphorylation (Figure 8A). Furthermore, p-Foxo3a immunostaining was markedly increased in STZ-induced diabetic rat glomeruli, and, interestingly, most of the p-FoxO3a staining and localization were cytoplasmic and not nuclear (Figure 8D). These results suggest that diabetic conditions, which usually are associated with increased TGF-β levels, may induce PI3K and Akt activation, leading to FoxO3a phosphorylation and its nuclear exclusion and inactivation.

We also tested cortical tissues from diabetic mice and found clear increases in p-Akt and p-FoxO3a levels in the 2-wk diabetic mice similar to rats (Figure 9A). These effects on Akt phosphorylation (approximately 10-fold) and FoxO3a phosphorylation (four-fold) were significantly greater in STZ-induced diabetic mouse cortex at 2 wk compared with control healthy mouse cortex (Figure 9, B and C, respectively). Unlike rat glomeruli, we noted a modest increase of FoxO1 phosphorylation in mouse cortical tissues (Figure 9A). Immunostaining of mouse cortex showed a marked increase of p-FoxO3a staining, and, similar to diabetic rat glomeruli, it mainly was cytoplasmic in nature.

We next examined whether these changes were sustained at 16 wk after the onset of diabetes in mice. It is interesting that no

**Table 1. TGF-β prevents mesangial cell apoptosis**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Nuclei Counted</th>
<th>Condensed Nuclei</th>
<th>P (χ² Test)</th>
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<tbody>
<tr>
<td>Control</td>
<td>117</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Serum depletion</td>
<td>127</td>
<td>13</td>
<td>0.002</td>
</tr>
<tr>
<td>TGF-β</td>
<td>120</td>
<td>7</td>
<td>0.02 versus serum depletion</td>
</tr>
</tbody>
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*aQuantification of apoptotic cells shown in Figure 6. Condensed nuclei were counted. In the absence of serum, a significant increase of condensed nuclei was observed compared with the normal condition (P = 0.002 versus serum + control). TGF-β treatment reduced the number of condensed nuclei (P = 0.02 versus serum depletion). No significant difference was observed between the numbers of condensed nuclei of control and TGF-β treatment (P > 0.05). These results suggest that TGF-β treatment prevents apoptosis of mesangial cells.*
clear differences in p-Akt or p-FoxO3a levels were observed between control and STZ-induced diabetic mouse cortex at 16 wk (data not shown). Therefore, Akt activation and FoxO3a phosphorylation may be key events in early DN but not in the later stages.

**Discussion**

Several lines of evidence have demonstrated that TGF-β plays a key role in the pathogenesis of DN. This has been attributed to multiple downstream effects of TGF-β. In this study, we provide evidence for a novel role of the PI3K/Akt/FoxO3a pathway in the MC dysfunction that is induced by TGF-β. TGF-β treatment of MC induced Akt activation and phosphorylation of FoxO3a at Ser253 (Akt) site but not at Ser644 (IKK site; Figure 1). Phosphorylation of FoxO3a by IKK has been related to malignancy or poor prognosis of cancer patients (43). Therefore, the regulation of FoxO3a in MC by TGF-β may be different from that in cancer cells. Akt most

**Figure 8.** Akt activation and FoxO3a phosphorylation in glomeruli from streptozotocin (STZ)-induced diabetic rats. (A) Immunoblotting analysis. Sieved glomeruli from healthy (control) and STZ-induced (STZ) diabetic rats were studied. Clear increase of Akt activation and FoxO3a phosphorylation (but not FoxO1) was observed in STZ-induced diabetic rats after 2 wk of diabetes. (B and C) Ratio of the p-Akt/total Akt (B) and p-FoxO3a/total FoxO3a (C) showed an eight-fold increase of p-Akt (P < 0.01) and a four-fold increase of p-FoxO3a (P < 0.05) in diabetic rats compared with control rats. (D) Immunohistochemistry of p-FoxO3a. Renal cortical sections from control or STZ-treated rats (STZ) were stained with anti–phospho253Ser-FoxO3a antibody (brown) and counterstained with hematoxylin (blue color). Strong extensive staining in cytoplasm was noted in STZ kidney with only very weak staining in control rat kidney.
likely is the kinase that phosphorylates FoxO3a in our study because we detected both Akt activation and serine 253 phosphorylation of FoxO3a in MC that were treated with TGF-β. In MC, the PI3K-Akt pathway is activated by TGF-β and implicated in the expression of collagen type 1 a2 gene (27) and also fibronectin expression (40) and thus also may mediate the profibrotic effects of TGF-β. TGF-β activates the PI3K/Akt pathway in several cell types (27,28,40), including MC. On the contrary, in hematopoietic cells, TGF-β family members induce apoptosis through expression of the phosphatase SHIP, which inhibits Akt activation (46). In this study, we noted that TGF-β activates Akt, phosphorylates and inactivates FoxO3a, decreases the expression of the pro-apoptotic gene Bim, and supports MC survival. Therefore, the effects of TGF-β via the PI3K pathway may be cell-type specific. It has been suggested that TGF-β activates the PI3K/Akt pathway via a direct interaction of TGF-β receptors and PI3K (29,30). Activation of TGF-β receptor serine threonine kinase can stimulate the PI3K/Akt pathway in MC (40). Our study supports the involvement of PI3K, because a PI3K inhibitor blocked TGF-β-induced Akt activation, FoxO3a phosphorylation, and FoxO transcriptional activity.
PDGF also has been shown to inactivate FoxO family proteins via Akt in MC (39).

Phosphorylation of FoxO3a causes nuclear exclusion and its association with 14-3-3. In the absence of survival factors, FoxO3a is dephosphorylated, translocated to nuclear, and activates target genes (32) that include the proapoptotic Bim gene (33–36). FoxO3a also protects cells from oxidant stress by up-regulating MnSOD (37). We observed that, along with FoxO3a phosphorylation in MC, TGF-β decreased the expression of Bim and MnSOD. It is interesting that we also observed that phosphorylation of Akt and FoxO3a were increased in glomeruli and cortical tissues of STZ-induced diabetic rats and mice. Because evidence shows that TGF-β levels are increased in these diabetic tissues (7,13,14), our data suggest that the results that are seen with TGF-β-treated MC in vitro reflect a similar diabetic renal disease situation in animal models.

It is interesting that we noted increases in FoxO3a phosphorylation in the STZ-injected mice only at 2 wk and not at 16 wk after the onset of diabetes. FoxO3a inactivation may be more related to the pathogenesis of early and not late stages of DN.

Because early stages of diabetes and DN are associated with MC accumulation and proliferation (along with hypertrophy) but not later stages, the downregulation of proapoptotic genes as a result of FoxO3a inactivation may be a key factor that mediates this MC survival in early DN. An increase in MC number has been noted in both type 1 and type 2 diabetic rodent models (47). Agents such as statins have been suggested to slow the progression of diabetic glomerulopathy by reducing MC proliferation and enhancing apoptosis (48). MC proliferation via cell-cycle changes can occur as a response to injury (49). Unopposed MC cell proliferation may account for rapid progression to ESRD. Although ECM deposition, fibrotic effects, and hypertrophy are major effects of agents that are involved in the pathogenesis of DN, they also can accelerate progression by contributing to MC survival. Our data now indicate that the downregulation of FoxO3a-dependent proapoptotic genes in early DN may be a key mechanism. In addition, the inactivation of FoxO3a was accompanied by a decrease in MnSOD expression. This can render the MC cells more sensitive to oxidant stress and thereby accelerate renal dysfunction that is associated with DN and other renal diseases.

In this study, TGF-β treatment also led to nuclear exclusion and cytoplasmic accumulation of FoxO3a in MC. TGF-β also decreased the transcriptional activity of FoxO. This was reversed by a PI3K inhibitor. This is consistent with the observation of decreased expression of the FoxO target genes Bim and MnSOD in TGF-β-treated MC. Cytoplasmic phospho-FoxO3a levels also were increased in STZ-induced diabetic rodent kidneys. TGF-β–induced decrease in Foxo transcriptional activity was abrogated by an Akt phosphorylation mutant of FoxO3a, further confirming that FoxO3a is a major player in TGF-β–mediated signaling via PI3K/Akt in MC.

We also noted that TGF-β can protect MC from serum depletion–induced apoptosis and hence act as a survival factor for MC at least under some conditions. This is supported by the data showing that TGF-β decreases the expression of the proapoptotic gene Bim via the PI3K/Akt pathway. It has been reported that the PI3K/Akt pathway can act as a survival and antiapoptotic signal in MC (50). Taken together, our results suggest that diabetic conditions and stimuli such as TGF-β can induce hyperphosphorylation and inactivation of FoxO3a, and this can lead to MC survival and oxidant stress. Tubulointerstitial disease also is a significant determinant of chronic kidney disease in diabetes. Because evidence shows that TGF-β–induced epithelial to mesenchymal transition is mediated by PI3K/Akt (28), the TGF-β/PI3K/Akt/FoxO3a pathway described in this study also may be relevant to the pathogenesis of tubulointerstitial disease that is associated with DN.

Conclusion

Several mediators, including Smads, p38, extracellular signal–regulated kinase, and MAPK are known major effectors of TGF-β (12,16,17,22–25). Our studies now add FoxO3a to this list as an important new effector that could be related to the pathogenic role of TGF-β in DN. Taken together, TGF-β signaling activates at least two pathways via PI3K/Akt (Figure 10). One pathway can enhance Smad3 or MAPK-mediated accumulation

![Figure 10. Mechanism of the pathogenesis of diabetic nephropathy mediated by TGF-β. Diabetic conditions can increase TGF-β expression in MC. TGF-β activates p38 mitogen-activated protein kinase, extracellular signal–regulated kinases, PI3K, Akt, and Smads and leads to an increase in extracellular matrix (ECM) genes such as collagen in MC. Cross-talk between Akt and Smad can augment collagen gene expression. Conversely, Akt activation by TGF-β also can induce FoxO3a phosphorylation and inactivation that results in decrease of Bim and MnSOD. This can result in increased cell survival and oxidant stress. Cooperation between these two TGF-β–regulated pathways, Smad (ECM accumulation) and FoxO3a (cell survival and oxidant stress), may accelerate kidney dysfunction.](image-url)
of ECM proteins, and the other pathway can decrease Bim and MnSOD expression via FoxO3a phosphorylation. These signaling events were noted not only in vitro in MC but also in vitro in rat and mouse models of diabetes, especially in the early stages. Cooperation between these two TGF-β-stimulated pathways can greatly augment ECM accumulation, cell survival, and oxidant stress. These new results suggest that Akt/FoxO pathway regulation may be a novel mechanism by which TGF-β can induce unopposed MC survival and oxidant stress in early DN, thereby accelerating renal disease.

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