TGF-β1 Protects against Mesangial Cell Apoptosis via Induction of Autophagy*

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Autophagy can lead to cell death in response to stress, but it can also act as a protective mechanism for cell survival. We show that TGF-β1 induces autophagy and protects mesangial cells from undergoing apoptosis during serum deprivation. Serum withdrawal rapidly induced autophagy within 1 h in mouse mesangial cells (MMC) as determined by increased microtubule-associated protein 1 light chain 3 (LC3) levels and punctate distribution of the autophagic vesicle-associated form LC3-II. We demonstrate that after 1 h there was a time-dependent decrease in LC3 levels that was accompanied by induction of apoptosis, evidenced by increases in cleaved caspase 3. However, treatment with TGF-β1 resulted in induction of the autophagy protein LC3 while suppressing caspase 3 activation. TGF-β1 failed to rescue MMC from serum deprivation-induced apoptosis upon knockdown of LC3 by siRNA and in MMC from LC3 null (LC3−/−) mice. We show that TGF-β1 induced autophagy through TAK1 and Akt activation, and inhibition of PI3K-Akt pathway by LY294002 or dominant-negative Akt suppressed LC3 levels and enhanced caspase 3 activation. TGF-β1 also up-regulated cyclin D1 and E protein levels while down-regulating p27, thus stimulating cell cycle progression. Bafilomycin A1, but not MG132, blocked TGF-β1 down-regulation of p27, suggesting that p27 levels were regulated through autophagy. Taken together, our data indicate that TGF-β1 rescues MMC from serum deprivation-induced apoptosis via induction of autophagy through activation of the Akt pathway. The autophagic process may constitute an adaptive mechanism to glomerular injury by inhibiting apoptosis and promoting mesangial cell survival.

In glomerular injury, counterbalancing of mesangial cell death and cell survival plays an important role in the pathogenesis of progressive glomerulosclerosis. Macautophagy, more commonly referred to as autophagy, is a fundamental cellular homeostatic process that cells use to degrade and recycle cellular proteins and remove damaged organelles, and its role has been implicated in the pathogenesis of some disease states including cancer and neurodegeneration (1–3). The process of autophagy involves the formation of double-membrane-bound vesicles called autophagosomes that envelope and sequester cytoplasmic components, including macromolecular aggregates and cellular organelles for bulk degradation by a lysosomal degradative pathway (4). A number of evolutionarily conserved autophagy-related (Atg) proteins have been identified to date. The mammalian homolog of Atg8, known as microtubule-associated protein 1 light chain 3 (LC3), functions at least in part as a structural component in the formation of autophagosomes (5). The best characterized form LC3B (hereafter referred to as LC3) is the most widely used autophagic marker (6). Autophagosomes fuse with lysosomes to form autolysosomes, in which the sequestered contents are degraded by lysosomal hydrolases into their basic components, such as amino acids and fatty acids, which are returned to the cytosol for recycling (7, 8).

Autophagy represents a genetically programmed process that is evolutionarily conserved in eukaryotes from yeast to mammal and has important roles in various biological events such as cellular remodeling during development and differentiation and adaptation to changing environmental conditions and lifespan extension (1). Excessive autophagic activity can lead to type II programmed cell death that is morphologically distinct from apoptosis or type I programmed cell death and thereby contribute to the pathology of diseases (9). However, an emerging body of evidence supports the notion that autophagy can promote cell survival, and the accumulation of autophagosomes may signify a survival response intended to rid cells of misfolded proteins or damaged organelles (10, 11). Indeed, autophagy is implicated to play a protective role against the progression of certain human diseases, including cancer, muscular disorders, and neurodegeneration, such as Huntington, Alzheimer, and Parkinson diseases (12, 13). Autophagy is also implicated in cellular defense mechanism to prevent infection by certain pathogenic bacteria and viruses (13–15). To date, the role of autophagy in the kidney remains largely under-investigated. The potential protective role of autophagy as a survival mechanism after cytotoxic injury to the kidney is suggested by a

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3 The abbreviations used are: LC3, light chain 3; MMC, mouse mesangial cell; mTOR, mammalian target of rapamycin; S6, S6 ribosomal protein; S6K, r70S6 kinase; TAK1, TGF-β-activated kinase 1; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; CA, constitutively active; DN, dominant negative; CDK, cyclin-dependent kinase.
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recent report showing that autophagy decreases apoptosis of renal tubular cells induced by cisplatin (16). Thus, autophagy may act as a major cytoprotective pathway promoting cell survival and play an essential function for tissue homeostasis. Transforming growth factor-β1 (TGF-β1) is a key mediator of glomerular injury response in progressive kidney diseases and regulates both cell growth and cell death. TGF-β1 acts as a multifunctional cytokine that regulates a number of biological responses including chemotaxis, cell cycle progression, differentiation, and apoptosis of target cells in a context- and cell-specific manner (17, 18). TGF-β1 has been shown to be a potent modulator of apoptosis in a variety of cell types, including epithelial cells, hepatocytes, hematopoietic cells, and lymphocytes (4, 19–21). The growth-inhibitory/pro-apoptotic effects of TGF-β1 have been well demonstrated in various cell types, including immune cells (22) and epithelial cells (23), consistent with its anti-inflammatory and tumor-suppressive functions. However, substantial evidence suggests that TGF-β1 also possesses the ability to inhibit apoptosis in some cell types, such as murine macrophages (24), human lung carcinoma cells (25), fibroblasts, myofibroblasts (5, 26–28), murine neurons (29), murine macrophages (24), human lung carcinoma cells (25), lung mesenchymal cells (30), and pulmonary artery endothelial cells (31). The effect of TGF-β1 on apoptosis and autophagy in primary mesangial cells has not been previously examined.

The Smad pathway represents a major signal transduction pathway activated by TGF-β superfamily ligands. Upon TGF-β1 stimulation, receptor-regulated (R)-Smads (Smad2 and Smad3) are activated and form complexes with the common mediator, Smad4. The R-Smad-Smad4 complex then translocates to the nucleus and functions as a transcriptional transactivator to regulate gene expression (32–34). In addition to the Smad pathway, there is a growing body of evidence that TGF-β1 signals through the activation of non-Smad signaling proteins, including TGF-β-activated kinase 1 (TAK1) and phosphatidylinositol 3-kinase 3-kinase (PI3K), and that there is a high degree of cell-type specificity of TGF-β1 signals (33, 35, 36). TAK1 triggers the activation of several downstream cell signaling cascades, including the MKK4/7-JNK and the MKK3/6-p38 MAPK (37, 38). We have previously reported that, in primary mouse mesangial cells (MCC), TAK1 mediates TGF-β1-induced activation of the MKK3-p38 signaling pathway (39, 40). Recent studies have also shown that TAK1 serves as an upstream activator of the PI3K-Akt pathway in hepatic macrophages and in osteoclasts (41, 42) and that TAK1-mediated Akt activation is essential for TGF-β1 to promote osteoclast survival (42). The PI3K-Akt pathway regulates a number of cellular processes including cell cycle progression and apoptosis (43). Cell cycle progression is controlled by cyclin/cyclin-dependent kinase (CDK) complexes and is negatively regulated by CDK inhibitors. Recent studies suggest that the PI3K-Akt pathay positively regulates G1/S cell cycle progression through increased cyclin D1 by inhibition of proteolytic degradation (44) and decreased p27Kip1 (p27) levels through ubiquitination-dependent proteolysis (45). The function and stability of p27 are regulated by its phosphorylation, and the main phosphorylation site on p27 is Ser-10. In early G1 phase, Ser-10 phosphorylation is increased and allows exportin CRM1 (chromosome region maintenance 1)-dependent export and KIP1 ubiquitylation-promoting complex-mediated proteolysis of cytoplasmic p27 (46).

The present study was undertaken to determine the functional role of TGF-β1 in the induction of autophagy for mesangial cell survival and protection from apoptosis induced by serum deprivation. We demonstrate that treatment with TGF-β1 resulted in the induction of LC3 protein levels and punctate distribution of the autophagic vesicle-associated form LC3-II, whereas suppressing the serum deprivation-induced caspase 3 activation and apoptosis in MMC. Moreover, we show that TGF-β1 induces autophagy via TAK1 and the PI3K-Akt dependent pathway. TGF-β1 failed to rescue the MMC from serum deprivation-induced apoptosis upon inhibition of autophagy by knockdown of LC3 by siRNA or by LC3 gene deletion. Our findings demonstrate the anti-apoptotic effects of TGF-β1 to rescue MMC from serum deprivation-induced apoptosis via induction of autophagy and promote mesangial cell survival.

MATERIALS AND METHODS

Reagents—Recombinant human TGF-β1 was obtained from R&D Systems (Minneapolis, MN). LY294002 was purchased from Cell Signaling Technologies (Beverly, MA). Bafilomycin A1, MG132, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma. LL-Z16409-2 was purchased from Enzo Life Sciences (Plymouth Meeting, PA). Polyclonal antibodies against caspase 3, cleaved caspase 3, p27, phospho-Akt (Ser-473), Akt, phospho-rubosomai protein S6 (Ser-235/236), and Smad4 were obtained from Cell Signaling Technologies (Beverly, MA). Antibodies against phospho-p27 (Ser-10), cyclin D1, cyclin E, β-actin, and horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-LC3 antibody was purchased from Nanotools (St. Louis, MO). Cy3-conjugated goat anti-mouse IgG antibody and normal goat serum were from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Lipofectamine Plus™ reagent was purchased from Invitrogen.

Cell Culture and Adenovirus Infection—MMC from male C57BL/6 mice, LC3 null (LC3−/−) mice, and wild-type (LC3+/+) littermate mice were isolated and characterized as previously described (47). The LC3−/− and LC3+/+ mice used for the isolation of MMC were kindly provided by Dr. M. Rabino-vitch (Stanford University, Stanford, CA). MMC established in culture were maintained in RPMI 1640 medium supplemented with 15% FBS, 100 units/ml of penicillin, and 100 µg/ml of streptomycin. Cells between 7 and 16 passages were used for the experiments. The triple-A mutant (K179A, T308A, and S473A), kinase dead dominant-negative Akt (Ad-Akt-DN), and constitutively active myr-Akt (Ad-Akt-CA) adenoviral vectors were generous gifts from Dr. K. Walsh (Boston University School of Medicine, Boston, MA). MMC grown to 70% confluence were infected for 24 h with Ad-Akt-CA or Ad-Akt-DN at a multiplicity of infection of 50.

MTT Assay—Cell viability was measured by the MTT assay. Briefly, cells were plated at 4 × 104 cells per well in 24-well tissue culture plates and incubated at 37 °C in a humidified atmosphere containing 5% CO2. The formazan product,
formed after 4 h of incubation with MTT (0.5 mg/ml), was dissolved in DMSO and read at 570 nm using a SpectraMax Plus microplate spectrophotometer (Molecular Devices Co., Sunnyvale, CA). Statistical significance of the experimental data from three independent experiments was determined by the Student’s *t* test, and *p* values <0.05 were considered significant.

Flow Cytometry—Apoptosis was measured by the detection of hypodiploid apoptotic nuclei, represented in sub-G1, by using flow cytometry. Briefly, 1 × 10⁶ MMC were suspended in 1 ml of PBS and centrifuged for 5 min at room temperature. The pellet was resuspended in 500 µl of PBS and fixed with 4.5 ml of 70% (v/v) cold ethanol for 1 h at 4 °C. The cells were then resuspended in 1 ml of cold PBS. After incubation for 30 min at 4 °C, the cells were centrifuged and resuspended in 1 ml of PBS solution containing RNase (50 µg/ml) and propidium iodide (60 µg/ml). After incubation for 30 min at 37 °C, the cells stained with propidium iodide were analyzed using BD LSRII flow cytometer (BD Biosciences) and FlowJo Version 7.2 software (Treestar Inc., Ashland, OR).

Western Blot Analysis—Cells were washed once with ice-cold PBS and lysed in buffer containing 1% Nonidet P-40, 20 mM Tris, (pH 8.0, 150 mM NaCl, 12.5 mM β-glycerophosphate, 1.5 mM MgCl₂, 2 mM EGTA, 1 mM NaF, 2 mM dithiothreitol, 1 mM sodium orthovanadate (Na₃VO₄), 1 mM phenylmethylsulfonyl fluoride, and 20 µg/ml aprotinin. Cells were disrupted by sonication on ice and then centrifuged for 15 min at 14,000 × g at 4 °C to remove cellular debris. The protein concentration was determined by BCA protein assay reagent kit (Pierce). For Western blotting, protein samples (20 µg) were subjected to SDS-PAGE and then transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% bovine serum albumin for 1 h and then incubated with primary antibodies overnight on a rocker at 4 °C. The membranes were washed three times (15 min/each) with TTBS buffer (10 mM Tris, pH 7.5, 50 mM NaCl, and 0.1% Tween 20) and then incubated with horseradish peroxidase-conjugated secondary antibodies for 30 min at room temperature. The target proteins were detected with LumiGLO (Cell Signaling Technologies). The results shown are representative of at least three independent experiments.

Immunofluorescence—MMC were plated onto Lab-Tek II dual chamber culture slides (Thermo Fisher Scientific, Rochester, NY), allowed to attach for 12 h, and then cultured in serum (15% FBS) or serum-free medium with or without exogenous TGF-β1 (10 ng/ml) for another 12 h. Immunofluorescence was performed as previously described (40). pEGFP-LC3B (GFP-LC3) construct was kindly provided by Dr. N. Mizushima (Tokyo Medical and Dental University, Tokyo, Japan). Transient transfection of expression vectors GFP-LC3 and control pEGFP-C1 (Clontech) was performed using Lipofectamine Plus™ (Invitrogen) according to the manufacturer’s instructions. In brief, cells grown to ~60% confluence on Lab-Tek II dual chamber slides were washed with phosphate-buffered saline (PBS) and with 60 ng of respective plasmids for 4 h under serum-free conditions. After transfection, cells were washed with PBS and incubated in medium supplemented with 15% FBS for 16 h. Cells were then incubated in serum (15% FBS) or serum-free medium with or without exogenous TGF-β1 (10 ng/ml) for another 12 h. Images were obtained using a Nikon D-eclipse C1 confocal fluorescence microscope. To measure autophagy, counting of GFP-LC3 puncta was used, as described by Klionsky et al. (48). A total of 50 cells per sample were examined at random. Results are presented as the mean ± S.D. of three independent experiments.

RT-PCR—Total RNA was isolated from MMC using Trizol® Reagent (Invitrogen), and 1 µg of total RNA was reverse-transcribed into the first strand cDNA in a reaction primed by oligo(dT) primer using Affinity Script Multipurpose Temperature cDNA Synthesis kit (Agilent Technologies) according to the manufacturer’s instructions. The first strand cDNA was used as the template for PCR of LC3B and GAPDH using Taq polymerase (Takara). The PCR primer sets for LC3B and GAPDH were prepared according to the corresponding DNA sequences of mouse LC3B (accession number, NM_026160; forward primer, 5'-CCATGCGTCCGAGAAAGCCTTC-3'; reverse primer, 5'-CACAGCCATTGTGCTCCGAATGTG-3') and GAPDH (accession number, BC145810; forward primer, 5'-GCATTGTGAGGCCCTCA-3'; reverse primer, 5'-GGGATAGAAACACGGAAGG-3'), respectively. PCR products were fractionated on agarose gel and visualized by ethidium bromide staining.

Small Interfering RNAs (siRNAs)—Knockdown of endogenous Smad4 with siRNA was carried out using DharmaFECT 4 (Dharmacon/Thermo Scientific) according to the manufacturer’s protocol. Briefly, MMC grown to 50–60% confluence in a 6-well plate were transfected with either Smad4 siRNA (ON-TARGETplus SMARTpool, L-004989, Dharmacon/Thermo Scientific), or control siRNA (ON-TARGETplus Non-Targeting Pool, D-001810, Dharmacon/Thermo Scientific). The final concentration of respective siRNA was 100 nm for each transfection. After incubating the cells for 5–7 h at 37 °C in a CO₂ incubator, the siRNA transfection mixture was replaced with normal growth medium. The experiments were carried out 48 h after transfection. Knockdown of endogenous LC3 with siRNA was carried out according to the Santa Cruz siRNA Transfection Protocol (Santa Cruz Biotechnology). Briefly, MMC grown to 50–80% confluence in a 6-well plate were transfected with either LC3B siRNA (sc-43391, Santa Cruz Biotechnology) or control siRNA (sc-37007, Santa Cruz Biotechnology). The final concentration of respective siRNA was 200 nm for each transfection, and the experiments were carried out 48 h after transfection.

Quantitation and Statistical Analysis—All experiments were performed at least three times. Densitometric analysis for the quantitation of Western blot and RT-PCR data was carried out by using Bio-Rad Quantity One software. Statistical significance of the experimental data from three independent experiments was determined by Student’s *t* test or analysis of variance, and *p* < 0.05 was considered significant.

RESULTS

TGF-β1 Abrogates Mesangial Cell Apoptosis Induced by Serum Deprivation—TGF-β1 has been shown to exert either pro-apoptotic or anti-apoptotic effects depending on the cell type or circumstances. Serum withdrawal is a potent inducer of apoptosis. We first examined the effects of TGF-β1 on the viability of MMC during serum deprivation by MTT assay. As
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shown in Fig. 1A, the withdrawal of serum led to a time-dependent decrease in mesangial cell viability compared with the cells maintained in medium containing 15% FBS. Treatment with TGF-β1 (10 ng/ml) attenuated these effects induced by serum deprivation. Furthermore, Western blot analysis demonstrated that serum withdrawal resulted in increased levels of cleaved forms of caspase 3, a marker of apoptosis, and treatment with TGF-β1 (10 ng/ml) significantly inhibited these effects induced by serum deprivation (Fig. 1B). To further confirm whether TGF-β1 treatment attenuates serum deprivation-induced apoptosis in MMC, flow cytometry was employed. As shown in Fig. 1C, serum deprivation resulted in increases in the number of sub-G1 apoptotic cells in a time-dependent manner, whereas TGF-β1 treatment significantly attenuated the increases in sub-G1 apoptotic cells. Thus, the increases in the serum deprivation-induced cleavage of caspase 3 were associated with increases in the number of sub-G1, apoptotic cells, and TGF-β1 stimulation protected MMC from serum deprivation-induced apoptosis. On the other hand, the protective effects of TGF-β1 against apoptosis of MMC were not observed when the addition of TGF-β1 was delayed up to 24 h after the start of serum deprivation, and TGF-β1 was unable to prevent serum deprivation-induced cleavage of procaspase 3 (Fig. 1D).

TGF-β1 Suppresses the Reduction of cyclin D1 and E and Induction of p27 under Serum Deprivation Conditions—We next assessed the effect of TGF-β1 on cyclins, the upstream regulators of cell cycle progression, in primary MMC. Cyclin D1 is responsible for cell cycle progression through the G1 phase, and cyclin E functions in the progression of the cell from late G1 to early S phase. As shown in Fig. 2A, decreases in the levels of both cyclin D1 and cyclin E were observed after serum deprivation in a time-dependent fashion compared with the cells maintained in medium containing 15% FBS, and treatment with TGF-β1 (10 ng/ml) attenuated these effects induced by serum deprivation. We also determined the effects of TGF-β1 on p27, an inhibitor of CDK that acts in G0 and G1 phases to inhibit cyclin E-Cdk2 and cyclin D-CDK complexes (49). As shown in Fig. 2B, the Ser-10-phosphorylated p27 and p27 protein levels were increased during serum deprivation, which was suppressed with treatment with TGF-β1.

TGF-β1 Induces Autophagic Marker, Microtubule-associated Protein 1 LC3—Conversion of cytosolic LC3-I to the lipidated form LC3-II conjugated to phosphatidylethanolamine is a widely used marker of autophagosomes (4, 5, 27). TGF-β1 regulates both cell growth and cell death. As shown in Fig. 3A, serum withdrawal rapidly induced the autophagic vesicle-associated form LC3-II within the first 1 h in MMC. We observed that beyond 1 h there was a time-dependent decrease in the levels of both LC3-I and LC3-II proteins in cells under sustained serum deprivation (Fig. 3B). On the other hand, treatment of TGF-β1 increased LC3-I as well as LC3-II to a lesser extent (Fig. 3B). Furthermore, an immunofluorescence experiment also demonstrated that TGF-β1 increased LC3 levels and punctate distribution (indicated by white arrowheads in Fig. 3C) of the autophagic vesicle-associated form LC3-II. Quanti-
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with bafilomycin A1 and TGF-
assess autophagic flux can be a more reliable method to moni-
tivation (see Fig. 3). GFP-LC3 is a genetically encoded probe that stabi-
LC3 turnover assay using selective inhibitor bafilomycin A1 to
associated-form LC3-II but also its subsequent degradation, the

Fig. 3

rivation (see Fig. 3).

A

| Serum deprivation (h) | TGF-β1 | Serum deprivation (h) | TGF-β1 |
|----------------------|--------|----------------------|--------|
| 4                    | -      | 4                    | -      |
| 8                    | +      | 8                    | +      |
| 12                   | +      | 12                   | +      |
| 24                   | +      | 24                   | +      |

Cyclin D1
Cyclin E
β-Actin

B

| Serum deprivation (h) | TGF-β1 | Serum deprivation (h) | TGF-β1 |
|----------------------|--------|----------------------|--------|
| 4                    | -      | 4                    | -      |
| 8                    | +      | 8                    | +      |
| 12                   | +      | 12                   | +      |
| 24                   | +      | 24                   | +      |

p-p27
p27
β-Actin

p27 phosphorylation of p27 at Ser-10 (Fig. 3). Western blot analysis shown in
Fig. 3A indicates that TGF-β1 treatment activated PI3K-Akt-mTOR signaling axis.

These data indicate that TGF-β1 treatments had increased numbers of puncta (20.1 ± 3.2) in
MMC compared with the number of puncta (12.6 ± 1.3) in untreated cells (see Fig. 3C) during serum depriva-
tivation (p < 0.05). Control cells in serum had 10.2 ± 1.0 puncta (d in Fig. 3C) per MMC. Because autophagy is a dynamic pro-
cess involving not only the induction of the autophagic vesicle-associated form LC3-II but also its subsequent degradation, the
LC3 turnover assay using selective inhibitor bafilomycin A1 to assess autophagic flux can be a more reliable method to moni-
tor autophagic activity (50). As shown in Fig. 3D, treatment with bafilomycin A1 and TGF-β1 significantly increased the
levels of LC3-II as compared with treatment with either TGF-β1 or bafilomycin A1 alone, indicating that autophagic flux is increased with TGF-β1 treatment under serum depriva-
tion, and therefore, the lower level of LC3-II may be a result of its autophagic degradation enhanced by TGF-β1 stimulation.
Moreover, we demonstrated that TGF-β1 increased LC3 mRNA expression in MMC, as shown in Fig. 3E. These data indicate that TGF-β1 stimulation induces LC3 expression at
the transcriptional and protein levels and is associated with autophagic activity.

TGF-β1-induced LC3 Expression Is Mediated via Non-Smad Pathway—we next explored the signal transducing pathways
involved in the induction of autophagy by TGF-β1. To deter-
mine whether the induction of LC3 is mediated through the
Smad pathway, we utilized siRNA directed against Smad4. In
MMC, knockdown of Smad4 did not affect TGF-β1-induced
LC3 protein expression either in the presence or absence of
bafilomycin A1 (Fig. 4A) or TGF-β1-induced LC3 mRNA expres-
sion (Fig. 4B). These results suggest that TGF-β1-induced
LC3 expression is mediated by non-Smad signaling path-
ways. Accordingly, treatment with LL-Z16409-2, a selective
inhibitor of TAK1 (51), suppressed TGF-β1-induced LC3 protein expres-
sion (Fig. 4C) as well as LC3 mRNA expression (Fig.
4D) in MMC. These data indicate an involvement of TAK1 in
mediating the induction of LC3 expression by TGF-β1 and pro-
vide further evidence that TGF-β1-induced LC3 expression occurs independent of the Smad signaling pathway.

PI3K-Akt-mTOR-S6K Signaling Axis Mediates TGF-β1-induced LC3 Expression—It has been previously shown that Akt
is recruited onto the plasma membrane through its specific
binding to phosphatidylinositol 3,4,5-trisphosphate, a product of PI3K, and is activated by phosphorylation. Thus, Akt phos-
phorylation has been used widely as an indicator of PI3K-Akt
pathway activation (17, 52–55). Western blot analysis shown in
Fig. 5A demonstrates that although serum deprivation led to a
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membrane through its specific binding to phosphatidylinosito
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**FIGURE 3.** TGF-β1 stimulation induces autophagy and LC3 expression. A, LC3-II is increased during early time points after serum deprivation, MMC were incubated in serum-free medium for the indicated time periods. Cell lysates were subjected to Western blot analysis with anti-LC3 antibody (upper panel), and expression levels of LC3-II protein were quantitated by densitometry (bottom panel); Western blotting for β-actin was used as the protein loading control. B, TGF-β1 increases LC3-I and LC3-II protein levels under prolonged serum deprivation. Total cell lysates from MMC incubated in serum (15% FBS) or serum-free medium with (+) or without (−) exogenous TGF-β1 for the indicated time periods were subjected to Western blot and densitometry analysis as indicated in A. All data are presented as the mean ± S.D. of three independent experiments. *, p < 0.05 versus control cells in serum; #, p < 0.05 versus cells under serum deprivation without TGF-β1 treatment at each time point. C, TGF-β1 increases immunofluorescence staining for endogenous LC3 and punctate distribution of ectopically expressed GFP-LC3. Immunofluorescence staining was performed as described under "Materials and Methods" to detect LC3 expression in MMC incubated in serum (a) or serum-free medium with (c) or without (b) TGF-β1 for 12 h and assessed by confocal fluorescence microscopy. MMC transiently transfected with GFP-LC3 were incubated in serum (d) or serum-free medium with (f) or without (e) TGF-β1 for 12 h. Bar = 20 μm. D, TGF-β1 enhances autophagy under prolonged serum deprivation. Total cell lysates from MMC incubated in serum-free medium with (+) or without (−) exogenous TGF-β1 (10 ng/ml) and with (+) or without (−) bafilomycin A1 (Baf) (100 nM) for 12 h were subjected to Western blot analysis using anti-LC3 antibody (upper panel), and expression levels of LC3-II protein were quantitated by densitometry (bottom panel). All data are presented as the mean ± S.D. of three independent experiments. #, p < 0.05 versus cells under serum deprivation without TGF-β1 treatment. E, TGF-β1 up-regulates LC3 mRNA expression in MMC under serum deprivation. Total RNA was isolated from MMC cultured in serum-free medium with (+) or without (−) exogenous TGF-β1 (10 ng/ml), and LC3B mRNA was measured after amplification by RT-PCR. Amplified cDNA level of LC3B was standardized with that of GAPDH, and quantitation is presented as the mean ± S.D. of three independent experiments. *, p < 0.05 versus without TGF-β1 treatment.

**DISCUSSION**

The balance between cell proliferation and cell death is central to many physiological processes, and its deregulation may induce disease. There is an emerging body of evidence indicating that autophagy is a major programmed mechanism.

depression-induced apoptosis of MMC. Thus, our findings suggest that the inhibition of autophagy utilizing three different strategies, namely silencing autophagy gene LC3 by siRNA or by the gene deletion and by inhibition of the autophagic process with bafilomycin A1, leads to apoptotic cell death and that TGF-β1 rescues mesangial cells from serum deprivation-induced apoptosis via induction of autophagy.

**TGF-β1 Suppresses Induction of p27 by Serum Deprivation via Autophagic Degradation Pathway**—Degradation of proteins is highly dependent on autophagy and the ubiquitin-proteasome system. To ascertain if the TGF-β1-suppression of p27 during serum deprivation was due to the induction of autophagy, we examined the effects of TGF-β1 on p27 levels in MMC from LC3−/− mice. As shown in Fig. 7A, treatment with TGF-β1 failed to down-regulate p27 protein levels and its phosphorylation at Ser-10 during serum deprivation, in contrast to the MMC from LC3+/+ mice, suggesting that TGF-β1-induced decrease in p27 is at least in part mediated by autophagy. Given that p27 is known to undergo the proteasomal degradation, we examined the effects of proteasome inhibitor MG132 and autophagic pathway inhibitor bafilomycin A1 on p27 levels. Fig. 7B demonstrates that treatment with bafilomycin A1, but not MG132, blocked TGF-β1-induced down-regulation of p27 protein during serum deprivation. These data suggest that autophagic degradation but not proteasomal degradation pathway contributes to the observed reduced levels of p27 protein by TGF-β1 during serum deprivation.
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It is generally thought that the anti-proliferative effects of TGF-β1 are mediated through repressing the transcription of CDK genes as well as inhibiting CDK activities associated with G1 to S phase progression through CDK inhibitors, resulting in cell growth arrest (58). Under serum deprivation conditions, however, our data provide evidence that TGF-β1 not only attenuates apoptosis of MMC but also promotes cell proliferation by inducing cell cycle progression rather than cell growth arrest. Serum deprivation severely reduced cell viability (Fig. 1A) through the induction of apoptosis, as indicated by caspase 3 activation (Fig. 1B), and increases in cells with hypodiploid apoptotic nuclei (Fig. 1C). We show that TGF-β1 treatment at the start of serum deprivation ameliorated these effects induced by serum deprivation. On the other hand, TGF-β1 was unable to inhibit caspase 3 activation if TGF-β1 treatment was delayed by its application 24 h after the start of serum deprivation (Fig. 1D). Thus, these data suggest that the protective effects of TGF-β1 against apoptosis by inhibiting caspase 3 activation might be mediated by affecting the early events in the apoptotic pathway. In addition to these anti-apoptotic effects of TGF-β1, our findings also provide evidence that under serum deprivation conditions TGF-β1 may promote MMC proliferation through the induction of cell cycle progression. It has been previously reported that in renal epithelial cells, mesangial cells, and several other cell types, TGF-β1 does not significantly alter cyclin-CDK levels (59–62) but, rather, inhibits the activity of CDKs through the kinase inhibitory protein (KIP) family of CDK inhibitors such as p27 and p21CIP1 (CIP, cyclin inhibitory protein) (p21) (59). However, our data show that TGF-β1 attenuated serum deprivation-induced reduction of cyclin D1 and cyclin E protein levels (Fig. 2A). Furthermore, serum deprivation increased not only the protein level of p27 (middle panel of Fig. 2B) but also the phosphorylation level of p27 at Ser-10 (upper panel of Fig. 2B), whereas these increases were abrogated by TGF-β1 treatment. Although both p27 and p21 have been previously shown to be regulated by TGF-β1, in quiescent mesangial cells p27 is constitutively expressed, whereas p21 is barely detected (63, 64). Similar differential expression of p27 and p21 is also observed in normal rat kidneys. Moreover, the onset of mesangial cell proliferation in the anti-Thy1.1 model was associated with a reduction of p27 levels (64), suggesting that p27 might be a predominant regulator of MMC proliferation. In this context, our data indicate that TGF-β1 promotes MMC proliferation under serum deprivation conditions through retaining the levels of cyclins and reducing the cyclin-dependent kinase inhibitor p27.

Autophagy constitutes a degradative mechanism for the removal and turnover of bulk cytoplasmic constituents via the endosomal-lysosomal system. During autophagy, the formation of autophagosomes involves redistribution of LC3 from the cytosol to autophagic vesicles with conversion of cytosolic LC3-I to phospholipid conjugate form LC3-II and is widely used to monitor autophagy (48). Our data demonstrate that LC3-II levels were increased during the early period (within 1 h) of serum deprivation in MMC, but thereafter the levels of both LC3-I and LC3-II were dramatically reduced under prolonged serum deprivation conditions (Fig. 3, A and B). TGF-β1 treatment, however, resulted in increased LC3-I and LC3-II levels.
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FIGURE 5. TGF-β1 induces autophagy via the PI3K-Akt-mTOR-S6K signaling axis. A, TGF-β1 induces Akt phosphorylation under serum deprivation. Total cell lysates isolated from MMC incubated in serum (15% FBS) or serum-free medium with (+) or without (−) exogenous TGF-β1 (10 ng/ml) for the indicated time periods were subjected to Western blot analysis with antibodies against phosphorylated Akt (p-Akt) and Akt. B, inhibition of PI3K-Akt-mTOR-S6K signaling axis by LY294002 suppresses LC3 protein expression and enhances the cleavage of caspase 3. MMC were pretreated with (+) or without (−) LY294002 (20 μM) for 1 h and then incubated in serum-free medium with (+) or without (−) exogenous TGF-β1 (10 ng/ml) for 12 h. Total cell lysates were isolated and subjected to Western blotting analysis with antibodies against Akt, p-Akt, ribosomal protein S6 (p-S6), LC3, cleaved caspase 3, and caspase 3. Western blotting for β-actin was used as the protein loading control. C, expression of dominant-negative mutant of Akt inhibits the expression of LC3-II protein and enhances the cleavage of caspase 3. MMC were infected with adenovirus expressing constitutively active Akt (CA) or dominant negative Akt (DN) followed by incubation in serum (15% FBS) or serum-free medium (SF) with (+) or without (−) exogenous TGF-β1 (10 ng/ml) for 12 h. Total cell lysates were isolated and subjected to Western blotting analysis with antibodies against p-Akt, Akt, LC3, cleaved caspase 3, and caspase 3. Western blotting for β-actin served as the loading control.

compared with the untreated cells under serum deprivation, as assessed by Western blotting (Fig. 3, A and B) and increased immunofluorescence staining of LC3 and the formation of autophagosomes indicated by punctate distribution of LC3 (Fig. 3C). We observed that the degree of increases in LC3-II protein levels was less than that of LC3-I protein with TGF-β1 treatment. There are two plausible explanations. First, it is possible that the influence of TGF-β1 stimulation with respect to the induction of LC3-I expression may be greater than its effect on the conjugation events after cleavage of LC3-I by Atg4, a cysteine protease responsible for the cleavage of the carboxyl terminus of LC3, to produce LC3-II (65) and thereby resulting in higher levels of LC3-I. Second, the increased autophagic flux by TGF-β1 stimulation subsequently would lead to rapid degradation of LC3-II itself that is bound to autophagosomes, therefore, resulting in the observed relatively lower levels of LC3-II protein by immunoblotting (50). We found that treatment with bafilomycin A1, an inhibitor of autophagosome-lysosome fusion, and TGF-β1 resulted in greater accumulation of LC3-II (Fig. 3D), indicating that the observed lower level of LC3-II (Fig. 3B) is likely from rapid degradation of LC3-II by TGF-β1 induced-autophagy. Recent reports have shown evidence for transcriptional regulation of certain autophagy genes, including LC3 and Atg12, in the induction of autophagy (66, 67). In our study, TGF-β1 increased LC3 mRNA expression during serum deprivation in MMC, demonstrating that TGF-β1 induces LC3 at the transcriptional level, and that this is accompanied by increases in both LC3-I and LC3-II protein expression (Fig. 3E). Moreover, our data demonstrate that in MMC the induction of LC3 expression by TGF-β1 is mediated by non-Smad signaling pathways, namely TAK1 (Fig. 4, C and D) and PI3K-Akt (Fig. 5, B and C) and is independent of the Smad signaling pathway (Fig. 4, A and B).

Few studies have previously reported that TGF-β1 induces autophagy in bovine mammary gland epithelial cells and neonatal piglet gut epithelium in the context that autophagy represents type II programmed cell death, which is complementary to apoptosis type of cell death induced by TGF-β1 treatment (68, 69). However, mechanisms for activation of autophagy by TGF-β1 and its role in renal cell growth and cell death have not been elucidated. Our data provide the first evidence that TGF-β1-induced autophagy plays a crucial role in TGF-β1-mediated mesangial cell survival under serum deprivation condition. Knockdown of LC3 in wild-type MMC by specific siRNA (Fig. 6A) or LC3 gene deletion in MMC from LC3 null (LC3−/−) mice (Fig. 6B) exhibited much higher caspase 3 activity compared with normal LC3 background under serum deprivation condition. More importantly, the protective effects of TGF-β1 against caspase 3 activation were drastically abrogated when LC3 expression was blocked. Furthermore, inhibition of autophagy by using bafilomycin A1 resulted in enhanced apoptosis and abrogated the protective effects of TGF-β1 against serum deprivation-induced apoptosis of MMC (Fig. 6C). Thus, our findings suggest that, at least in part, TGF-β1-induced autophagy mediates the protective effects of TGF-β1 against serum deprivation-induced mesangial cell apoptosis.

We and others have demonstrated that TGF-β1 activates Akt in various cell types including MMC (70). In MMC, we found that serum deprivation rapidly reduced phosphorylated Akt, whereas TGF-β1 stimulation up-regulated Akt phosphorylation in a time-dependent manner (Fig. 5A). Recent studies have reported that activation of Akt regulates the expression and phosphorylation of p27 (71, 72). In glioblastoma tumor cells, activated PI3K and elevated phosphorylated Akt levels were associated with reduced p27 levels, and inhibition of the PI3K-Akt pathway, either by a selective chemical inhibitor LY294002 or the expression of a kinase dead dominant-nega-
sub-G1 fraction is shown. Data are presented as the mean ± S.D. of three independent experiments. *, p < 0.05 versus cells transfected with control siRNA. LC3 siRNA (+) or control siRNA (−) transfected MMC incubated in serum-free medium with (+) or without (−) exogenous TGF-β1 (10 ng/ml) for 12 h were analyzed by Western blot using anti-cleaved caspase 3 and anti-caspase 3 antibodies (bottom panel). Immunoblotting with anti-β-actin antibody was used as the protein loading control. B, LC3 gene deletion abolishes TGF-β1-induced suppression of caspase 3 activation by serum deprivation. Total cell lysates were isolated from LC3 null (LC3−/−) MMC and wild-type (LC3+/+) littermate MMC incubated in serum (15% FBS) or serum-free medium (SF) with (+) or without (−) exogenous TGF-β1 (10 ng/ml) for 12 h and subjected to Western blot analysis with antibody against p27 (upper panel), and expression levels of p27 protein were quantitated by densitometry (bottom panel). All data are presented as the mean ± S.D. of three independent experiments. *, p < 0.05 versus cells under serum deprivation alone. Western blotting with anti-β-actin antibody was used as the protein loading control.

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FIGURE 6. TGF-β1 abrogates serum deprivation-induced apoptosis via LC3 activation. A, siRNA-mediated knockdown of LC3 prevents TGF-β1-induced suppression of caspase 3 activation by serum deprivation. MMC transfected with LC3 specific siRNA (+) or control siRNA (−) were analyzed by Western blot with anti-LC3 antibody (upper left panel), and expression levels of LC3 protein were quantitated by densitometry (upper right panel). Data are presented as the mean ± S.D. of three independent experiments. *, p < 0.05 versus cells transfected with control siRNA. LC3 siRNA (+) or control siRNA (−) transfected MMC incubated in serum-free medium with (+) or without (−) exogenous TGF-β1 (10 ng/ml) for 12 h were analyzed by Western blot using anti-cleaved caspase 3 and anti-caspase 3 antibodies (bottom panel). Immunoblotting with anti-β-actin antibody was used as the protein loading control. B, LC3 gene deletion abolishes TGF-β1-induced suppression of caspase 3 activation by serum deprivation. Total cell lysates were isolated from LC3 null (LC3−/−) MMC and wild-type (LC3+/+) littermate MMC incubated in serum (15% FBS) or serum-free medium (SF) with (+) or without (−) exogenous TGF-β1 (10 ng/ml) for 12 h and subjected to Western blot analysis with antibody against p27 (upper panel), and expression levels of p27 protein were quantitated by densitometry (bottom panel). All data are presented as the mean ± S.D. of three independent experiments. *, p < 0.05 versus cells under serum deprivation alone. Western blotting with anti-β-actin antibody was used as the protein loading control.

TGF-β1 down-regulates p27 during serum deprivation via autophagic degradation pathway. A, LC3 gene deletion abrogates TGF-β1-induced down-regulation of p27. Total cell lysates were isolated from LC3 null (LC3−/−) MMC and wild-type (LC3+/+) littermate MMC incubated in serum (15% FBS) or serum-free medium (SF) with (+) or without (−) exogenous TGF-β1 (10 ng/ml) for 12 h and subjected to Western blot analysis with anti-cleaved caspase 3 and anti-caspase 3 antibodies (upper left panel), and expression levels of p27 protein were quantitated by densitometry (bottom panel). All data are presented as the mean ± S.D. of three independent experiments. *, p < 0.05 versus cells under serum deprivation alone. Western blotting with anti-β-actin antibody was used as the protein loading control.

Blockade of the PI3K pathway by its selective inhibitor LY294002 abrogated the activation of the PI3K-Akt-mTOR-
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S6K signaling axis, resulting in increased caspase 3 activation and reduction of LC3 expression regardless of TGF-β1 stimulation (Fig. 5B). Although we cannot rule out the potential negative effects of LY294002 on the expression of LC3 through the inhibition of class III PI3K complex (Vps34-Vps15-Beclin-1), known to be an autophagy inducer, our data indicate that the PI3K-Akt-mTOR-S6K signaling axis plays a pivotal role in TGF-β1-induced up-regulation of LC3 expression and inhibition of caspase 3 activation. We further confirmed our findings by specific blockade of Akt employing the expression of a dominant-negative mutant of Akt, which shows similar effects on LC3 expression and caspase 3 activation as treatment with LY294002 (Fig. 5C). Previous reports have shown that, in general, Akt activation inhibits autophagy through mTOR activation in various cell types (73–76), whereas our data suggest that in the context of serum deprivation conditions, TGF-β1-induced Akt activation induces autophagy in MMC. Indeed, our findings are supported by the more recent studies demonstrating that PI3K-Akt pathway can also positively regulate autophagy in HeLa cells (77) and NB4 promyelocytic leukemia cells (78). In addition, it has been previously reported that mTOR-S6K positively regulates 6-thioguanine-induced autophagy (79). Interestingly, a recent report by Hu et al. (80) indicates that PI3K-dependent induction of autophagy is also a tool used by the eukaryotic pathogen Cryptococcus neoformans to ensure survival and effect host cell damage during infection.

The cyclin-dependent kinase inhibitor p27 has been shown to be a critical link between induction of autophagy and inhibition of apoptosis in response to nutrient depletion (81). However, the mechanisms implicated in linking cell cycle progression and autophagy regulation are not yet known. We have shown that TGF-β1 stimulation strongly reduces p27 levels in wild-type MMC. Ubiquitination-dependent proteasomal degradation of p27 is well documented and shown to be regulated by different pathways (46, 82, 83). In general, although proteasomal degradation of short-lived proteins regulates cellular function of the protein as well as maintaining amino acid levels under normal conditions, during an extended period of nutrient starvation de novo synthesis and proteasomal degradation do not occur because of the lack of substrates and energy, and the vital amino acids are produced through autophagy (84). Our data demonstrate that either LC3 gene deletion or inhibition of autophagy with bafilomycin A1 showed no significant reduction of p27 level by TGF-β1 stimulation under the same serum deprivation conditions (Fig. 7). Moreover, blocking the ubiquitination-dependent proteasomal degradation by treatment with MG132 did not alter the p27 levels with or without TGF-β1 stimulation. These findings indicate that p27 is degraded through autophagy-mediated lysosomal degradation rather than ubiquitination-dependent proteasomal degradation pathway under serum deprivation.

In summary, we demonstrate that TGF-β1-induced autophagy protects glomerular mesangial cells from undergoing apoptosis under serum deprivation conditions through activation of the PI3K-Akt-mTOR-S6K signaling axis (Fig. 8). This is the first evidence that TGF-β1-induced Akt activation up-regulates autophagy and enhances cell cycle progression under serum deprivation conditions through regulating the protein levels of cyclins and cyclin-dependent kinase inhibitor p27 to promote mesangial cell survival.

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