Autophagy Regulates TGF-β Expression and Suppresses Kidney Fibrosis Induced by Unilateral Ureteral Obstruction

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ABSTRACT
Autophagy is an evolutionarily conserved process that cells use to degrade and recycle cellular proteins and remove damaged organelles. During the past decade, there has been a growing interest in defining the basic cellular mechanism of autophagy and its roles in health and disease. However, the functional role of autophagy in kidney fibrosis remains poorly understood. Here, using GFP-LC3 transgenic mice, we show that autophagy is induced in renal tubular epithelial cells (RTECs) of obstructed kidneys after unilateral ureteral obstruction (UUO). Deletion of LC3B (LC3−/− mice) resulted in increased collagen deposition and increased mature profibrotic factor TGF-β levels in obstructed kidneys. Beclin 1 heterozygous (beclin 1+/-) mice also displayed increased collagen deposition in the obstructed kidneys after UUO. We also show that TGF-β1 induces autophagy in primary mouse RTECs and human renal proximal tubular epithelial (HK-2) cells. LC3 deficiency resulted in increased levels of mature TGF-β in primary RTECs. Under conditions of TGF-β1 stimulation and autoinduction, inhibition of autolysosomal protein degradation by bafilomycin A1 increased mature TGF-β protein levels without alterations in TGF-β1 mRNA. These data suggest a novel intracellular mechanism by which mature TGF-β1 protein levels may be regulated in RTECs through autophagic degradation, which suppresses kidney fibrosis induced by UUO. The dual functions of TGF-β1, as an inducer of TGF-β1 autoinduction and an inducer of autophagy and TGF-β degradation, underscore the multifunctionality of TGF-β1.


In the kidney, fibrosis is responsible for chronic progressive kidney failure, and the prevalence of CKD is increasing worldwide.1,2 Extracellular matrix (ECM) protein production and progressive accumulation are hallmarks of renal tubulointerstitial fibrosis in progressive kidney disease. Collagens are the main components of the ECM in the kidney, and type I collagen (Col-I) is the major type associated with disease states.3,4 The cellular mechanisms that facilitate tubulointerstitial fibrosis after injury remain incompletely defined. Recent lineage tracing or genetic fate mapping studies have strongly challenged the theory that renal tubular epithelial cells (RTECs) traverse the tubular basement membrane to become myofibroblasts in a process of epithelial-to-mesenchymal transition (EMT), but rather, that interstitial pericytes/perivascular fibroblasts are the myofibroblast progenitor cells.5–7 It also has been proposed that profibrotic factors, such as TGF-β1, are upregulated in the tubular interstitial area on injury, leading to kidney fibrosis.8 TGF-β1 induces production of ECM proteins, including fibronectin and collagens, and inhibits degradation of...
ECM proteins mainly by matrix metalloproteinases.\textsuperscript{9–11} Given the recent evidence that casts doubts about the role of EMT \textit{in vivo}, how RTECs contribute to the development of renal tubulo-interstitial fibrosis is not entirely clear.

TGF-\(\beta\) is synthesized as a single polypeptide precursor that includes a preregion signal peptide, which is removed by proteolytic cleavage, and pro-TGF-\(\beta\), containing a preregion called the latency-associated peptide and a mature TGF-\(\beta\), and it converts to homodimeric pro-TGF-\(\beta\) through disulfide bonds.\textsuperscript{12} After cleavage by proprotein convertases, such as furin, latency-associated peptide remains noncovalently associated with the dimeric form of mature TGF-\(\beta\) as the small latent complex (SLC).\textsuperscript{13} SLC formation occurs in the Golgi apparatus, and mature TGF-\(\beta\) is secreted as part of SLC and associated with latent TGF-\(\beta\)--binding protein to form TGF-\(\beta\) large latent complex, which interacts with ECM. On stimulus, the dimeric form of mature TGF-\(\beta\) is dissociated from large latent complex and becomes the bioactive mature TGF-\(\beta\) ligand, which can then bind TGF-\(\beta\) receptors to trigger downstream Smad-dependent or -independent signaling pathways.\textsuperscript{12,13} Thus, the availability of mature TGF-\(\beta\) is the limiting factor of TGF-\(\beta\) activity and not TGF-\(\beta\) synthesis per se, because the body generates more pro-TGF-\(\beta\) than necessary. Whereas TGF-\(\beta\)/TGF-\(\beta\) receptor downstream signaling pathways have been extensively investigated, the regulation of TGF-\(\beta\) maturation and bioavailability has not been well studied but may serve as an important target for fibrotic diseases that alter TGF-\(\beta\) signaling.

Macroautophagy, hereafter referred to as autophagy, is a fundamental cellular homeostatic process that cells use to degrade and recycle cellular proteins and remove damaged organelles. The process of autophagy involves the formation of double membrane–bound vesicles called autophagosomes that envelop and sequester cytoplasmic components, including macromolecular aggregates and cellular organelles, for bulk degradation by a lysosomal degradative pathway.\textsuperscript{14} Autophagy can be induced in response to either intracellular or extracellular factors, such as amino acid or growth factor deprivation, hypoxia, low cellular energy state, endoplasmic reticulum stress or oxidative stress, organelle damage, and pathogen infection.\textsuperscript{15–22} To date, over 30 genes involved in autophagy have been identified in yeast, and they have been termed autophagy-related genes (Atgs). The mammalian ortholog of Atg8 is comprised of a family of proteins known as microtubule-associated protein 1 light chain 3 (LC3) that functions as a structural component in the formation of autophagosomes.\textsuperscript{23} LC3B (herein referred to as LC3) is the best characterized form and the most widely used as an autophagic marker. The conversion of the cystolic form of LC3 (LC3-I) to lipiddated form (LC3-II) indicates autophagosome formation. In contrast to LC3, Beclin 1, encoded by the beclin 1 gene, is the mammalian ortholog of yeast Atg6 that is required for the initiation of autophagy through its interaction with Vps34. Homozygous deletion of beclin 1 (beclin 1\textsuperscript{+/−}) exhibits early embryonic lethality, whereas heterozygous deletion (beclin 1\textsuperscript{+/-}) results in increased incidence of spontaneous tumorigenesis, abnormal proliferation of mammary epithelial cells and germinal center B lymphocytes, and increased susceptibility to neurodegeneration.\textsuperscript{24–27}

We previously reported that autophagy promotes intracellular degradation of Col-I induced by TGF-\(\beta\)1 in glomerular mesangial cells.\textsuperscript{28} In the present study, we explored the functional role of autophagy in an \textit{in vivo} model of progressive kidney fibrosis induced by unilateral ureteral obstruction (UUO) in autophagy-deficient LC3 null (LC3\textsuperscript{−/−}) and heterozygous (beclin 1\textsuperscript{+/-}) mice and green fluorescent protein (GFP)-LC3 transgenic mice. We also performed functional studies in primary cultured mouse RTECs and human renal proximal tubular epithelial (HK-2) cells. We hypothesized that induction of autophagy in RTECs promotes TGF-\(\beta\) degradation and thereby reduces TGF-\(\beta\) secretion and suppresses development of kidney fibrosis.

RESULTS

Autophagy Is Induced Primarily in RTECs of Obstructed Kidneys after UUO

We had previously reported that, in glomerular mesangial cells, autophagy promotes intracellular degradation of collagen induced by TGF-\(\beta\)1.\textsuperscript{28} To investigate the functional role of autophagy in kidney fibrosis, we examined the induction of autophagy in the kidney after injury induced by UUO in mice. We first determined the expression of two autophagy-related proteins, Beclin 1 and LC3, in both obstructed and contralateral kidneys at 3 and 7 days after UUO and compared it with sham-operated kidneys. As shown in Figure 1A, the levels of Beclin 1, LC3-I, and LC3-II proteins were increased in the obstructed kidneys at 3 and 7 days after UUO but not the corresponding contralateral kidneys compared with sham-operated kidneys. Using GFP-LC3 transgenic mice, we examined the functional role of autophagy in an \textit{in vivo} model of progressive kidney fibrosis.\textsuperscript{29} We previously reported that autophagy promotes intracellular degradation of collagen induced by TGF-\(\beta\)1.\textsuperscript{28} To examine overall cellular distribution of the autophagosome marker LC3 in the kidneys of mice subjected to UUO for 3, 7, 10, and 14 days. Punctate distribution of GFP-LC3 was visualized by confocal fluorescence microscopy, and representative images are shown in Figure 1B. We observed a high level of constitutive autophagy in the glomeruli under basal conditions, consistent with a previous report in podocytes compared with other intrinsic renal cells\textsuperscript{30} that did not change after UUO (data not shown). In contrast, there was little constitutive autophagy within the renal tubules. However, after UUO, the abundance of GFP-LC3 puncta (Figure 1B, green dots) in RTECs of obstructed kidneys increased, but it did not increase in contralateral kidneys compared with sham-operated kidneys. We also used \(\alpha\)-smooth muscle actin (\(\alpha\)-SMA) as an interstitial myofibroblast marker to examine whether autophagosomes can be observed in myofibroblasts after UUO. As shown in Figure 1B, an increased number of \(\alpha\)-SMA–expressing cells (Figure 1B, red) was detected in the interstitium of the
broid, we examined the kidneys of LC3 null (LC3\(^{-/-}\)) mice and LC3\(^{+/+}\) littermates.25,33 As shown in Figure 2A, deletion of LC3 expression, a marker of apoptosis, compared with LC3\(^{+/+}\) mice. The number of terminal deoxynucleotidyl transferase-mediated digoxigenin-deoxyuridine nick-end labeling (TUNEL)-positive cells was also significantly greater in the obstructed kidneys of LC3\(^{-/-}\) mice, indicating that tubular epithelial cell apoptosis was significantly increased at 7 days after UUO injury compared with LC3\(^{+/+}\) mice (Figure 3B). In contrast, there were no significant differences in the number of TUNEL-positive cells in the obstructed kidneys of LC3\(^{-/-}\) mice compared with LC3\(^{+/+}\) mice at 7 days after UUO (Figure 3C). Thus, protein expression in the 7-day UUO kidney compared with LC3\(^{+/+}\) mice and sham-operated LC3\(^{-/-}\) mice (Figure 2C). Consistently, Masson Trichrome staining revealed that LC3\(^{-/-}\) mice developed increased collagen deposition in the kidneys 7 days after UUO compared with sham-operated LC3\(^{-/-}\) mice and LC3\(^{+/+}\) control mice (Figure 2D). Similarly, mice deficient in autophagic protein Beclin 1 through heterozygous deletion (beclin 1\(^{-/-}\)) also exhibited increased collagen deposition, confirming our previous report that reduced Beclin 1 expression leads to increased collagen levels and profibrotic phenotype.28 Here, we also show that the beclin 1\(^{-/-}\) mice developed increased collagen deposition in the kidneys 7 days after UUO compared with sham-operated beclin 1\(^{+/+}\) mice and beclin 1\(^{+/+}\) control mice (Figure 2E). Taken together, these data showed that deficiency of LC3 or Beclin 1 leads to increased collagen protein levels, suggesting that LC3 and Beclin 1 may function to inhibit kidney fibrosis in the obstructed kidneys.

**Deficiency of Autophagic Proteins LC3 and Beclin 1 Results in Increased Collagen Deposition in Obstructed Kidneys**

To assess the function of the autophagy-related protein LC3 in kidney fibrosis, we examined the kidneys of LC3 null (LC3\(^{-/-}\)) mice compared with wild-type littermate (LC3\(^{+/+}\)) mice 3 and 7 days after UUO. We first confirmed the deletion of LC3 expression in the kidneys from LC3\(^{-/-}\) mice by Western blot analysis (Figure 2A). Next, immunofluorescence staining for Col-1 revealed that collagen accumulation was significantly enhanced in the tubulointerstitial areas of kidneys of LC3\(^{-/-}\) mice at 7 days after UUO compared with sham-operated LC3\(^{-/-}\) mice and LC3\(^{+/+}\) control mice (Figure 2B). Western blot analysis further confirmed the findings that disruption of LC3-enhanced Col-I protein expression in the 7-day UUO kidney compared with LC3\(^{+/+}\) mice and sham-operated LC3\(^{-/-}\) mice (Figure 2C). Consistently, Masson Trichrome staining revealed that LC3\(^{-/-}\) mice developed increased collagen deposition in the kidneys 7 days after UUO compared with sham-operated LC3\(^{-/-}\) mice and LC3\(^{+/+}\) control mice (Figure 2D). Similarly, mice deficient in autophagic protein Beclin 1 through heterozygous deletion (beclin 1\(^{-/-}\)) also exhibited increased collagen deposition, confirming our previous report that reduced Beclin 1 expression leads to increased collagen levels and profibrotic phenotype.28 Here, we also show that the beclin 1\(^{-/-}\) mice developed increased collagen deposition in the kidneys 7 days after UUO compared with sham-operated beclin 1\(^{+/+}\) mice and beclin 1\(^{+/+}\) control mice (Figure 2E). Taken together, these data showed that deficiency of LC3 or Beclin 1 leads to increased collagen protein levels, suggesting that LC3 and Beclin 1 may function to inhibit kidney fibrosis in the obstructed kidneys.

**RTEC Apoptosis Is Enhanced in Obstructed Kidneys of beclin 1\(^{+/+}\) Mice**

There is emerging evidence indicating that Beclin 1 regulates crosstalk between apoptosis and autophagy and has an antiapoptotic role in several settings, including chemotherapy, irradiation, immunotherapy, angiogenesis inhibitors, nutrient deprivation, and hypoxia.31 It has been previously shown that apoptosis is increased in obstructed renal tubules.32 However, the functional role of Beclin 1 and LC3 in UUO-induced kidney injury and apoptosis has not been shown. Therefore, we examined whether deficiency of Beclin 1 or LC3 influences RTEC apoptosis in the UUO kidney. Although the beclin 1\(^{-/-}\) mice are embryonic lethal, the beclin 1\(^{+/+}\) mice are viable and display approximately 50% reduction in autophagic activity compared with beclin 1\(^{+/+}\) littermates.25,33 As shown in Figure 3A, increased apoptosis in the obstructed kidneys of beclin 1\(^{-/-}\) mice was revealed by higher levels of cleaved caspase-3 expression, a marker of apoptosis, compared with beclin 1\(^{+/+}\) mice. The number of terminal deoxynucleotidyl transferase-mediated digoxigenin-deoxyuridine nick-end labeling (TUNEL)-positive cells was also significantly greater in the obstructed kidneys of beclin 1\(^{-/-}\) mice, indicating that tubular epithelial cell apoptosis was significantly increased at 7 days after UUO injury compared with beclin 1\(^{+/+}\) mice (Figure 3B). In contrast, there were no significant differences in the number of TUNEL-positive cells in the obstructed kidneys of LC3\(^{-/-}\) mice compared with LC3\(^{+/+}\) mice at 7 days after UUO (Figure 3C). Thus,
these data suggest that Beclin 1 functions to protect RTECs from apoptosis after UUO injury.

**TGF-β1 Induces Autophagy in Cultured RTECs**

TGF-β1 has been reported to be upregulated in response to injurious stimuli, including UUO, and mediates synthesis of ECM proteins and kidney fibrosis.8 Our findings shown above indicate that autophagy is primarily induced in tubular epithelial cells of obstructed kidneys after UUO injury and that LC3 deficiency promotes kidney fibrosis in vivo. We next assessed whether mature TGF-β but not pro-TGF-β levels were significantly increased in the obstructed kidneys of LC3−/− mice compared with wild-type LC3+/+ mice (Figure 5) after UUO, indicating that LC3 functions to suppress mature TGF-β levels induced by UUO.

**Mature TGF-β Is Degraded by Autophagic Pathway under TGF-β1 Stimulation and Autoinduction**

Autoinduction of TGF-β1 mRNA by TGF-β1 has been shown in RTECs and other cell types, such as mesangial cells,37,38 We...
shown in Figure 6A, TGF-β protein levels were further enhanced. These findings indicate that disruption of LC3 leads to increased mature TGF-β protein levels under TGF-β stimulation and autoinduction in RTECs. We further examined whether autophagy function regulates the level of mature TGF-β levels in HK-2 cells by the inhibition of autolysosomal degradation using bafilomycin A1.39,40 As shown in Figure 6B, mature TGF-β protein levels were significantly increased in HK-2 cells by TGF-β1 stimulation (Figure 6B, middle panel, lane 2) and further increased on cotreatment with bafilomycin A1 (Figure 6B, middle panel, lane 4). Thus, these data suggest that the autolysosomal protein degradation pathway may be responsible for the intracellular degradation of mature TGF-β protein.

To assure that the increases in mature TGF-β protein levels observed with bafilomycin A1 treatment were not because of corresponding increases in TGF-β1 mRNA expression and confirm the TGF-β autoinduction in RTECs, we performed semiquantitative RT-PCR, and representative results are shown in Figure 6C. No significant increases in the levels of TGF-β1 mRNA were detected in HK-2 cells on treatment with bafilomycin A1 compared with untreated cells (Figure 6C, lane 3 versus lane 1). Stimulation with TGF-β1 significantly increased TGF-β1 mRNA, confirming TGF-β autoinduction in RTECs as expected, but cotreatment with bafilomycin A1 did not further increase TGF-β1 mRNA (Figure 6C, lane 2 versus lane 4). Thus, inhibition of autophagy increased mature TGF-β1 protein levels without similarly inducing TGF-β1 mRNA expression, indicating that the increases in mature TGF-β protein levels are a result of decreased degradation rather than increased synthesis.

We further determined whether intracellular TGF-β1 is colocalized with LC3 puncta, which represent autophagosome formation.36 We assessed for punctate distribution of LC3 (Figure 6D, red) and subcellular localization of TGF-β1 (Figure 6D, green) visualized by confocal fluorescence microscopy, and representative images are shown in Figure 6D. We observed that treatment with bafilomycin A1 alone or TGF-β1 increased the abundance of autophagosomes (Figure 6D, red dots) compared with the nonstimulated control cells. These results are
consistent with the Western blot analysis (Figure 4A) detecting increases in endogenous LC3-II. We also observed that cotreatment with bafilomycin A1 and TGF-β1 increased the TGF-β1 staining (Figure 6D, green) compared with bafilomycin A1 or TGF-β1 treatment alone or nonstimulated control cells. Moreover, cotreatment with bafilomycin A1 and TGF-β1 resulted in increased colocalization of LC3 and TGF-β1 (seen as orange-colored dots in the merged images in Figure 6D) compared with bafilomycin A1 or TGF-β1 treatment alone or nonstimulated control cells.

Increases in mature TGF-β lead to increased TGF-β secretion, and we examined whether conditioned media from TGF-β1–treated LC3−/− RTECs enhance Col-I production in NIH3T3 fibroblasts in vitro. These data suggest that LC3 may negatively regulate the level of mature TGF-β in RTECs and that LC3 deficiency enhances the level of secreted active TGF-β that leads to increase in collagen production by adjacent interstitial fibroblasts (Figure 7).

DISCUSSION

In the present study, we investigated the functional role of autophagy in kidney injury and fibrosis. We show that kidney
injury induced by UUO, a well-established *in vivo* model of progressive kidney fibrosis, results in induction of autophagy and that deficiency of autophagic protein LC3 leads to increased collagen deposition and mature TGF-β levels in obstructed kidneys. Our findings suggest that autophagy regulates TGF-β expression and suppresses kidney fibrosis.

An emerging body of evidence suggests that the induction of autophagy constitutes a cytoprotective mechanism as a stress-adaptive response to tissue injury and that dysregulation of autophagy leads to the pathogenesis of human diseases. In the kidney, it has been shown that podocytes, which are terminally differentiated epithelial cells lining the outer aspect of the glomerular basement membrane, exhibit a high basal level of autophagy, which is thought to represent a self-repair/survival mechanism that is especially important in postmitotic cells, such as podocytes, that, like neurons, have a very limited capacity for cell division and replacement. In contrast, our studies using GFP-LC3 transgenic mice show

### Figure 5

Autophagy suppresses mature TGF-β levels in the kidneys after UUO. Expression of pro-TGF-β and mature TGF-β proteins in the kidney lysates from LC3+/+ and LC3−/− mice 7 days after UUO or sham operation. Representative Western blot data from two different mice in each group are shown. β-Actin was used as a loading control. The relative abundance of mature TGF-β was quantitated as the ratio of mature TGF-β to β-actin by densitometry, and data are presented as the mean±SEM of three independent experiments. *P<0.01 versus sham mice; **P<0.05 versus LC3+/+ mice with UUO.

### Figure 6

Autophagy promotes degradation of mature TGF-β in RTECs. (A) Levels of pro–TGF-β and mature TGF-β in primary cultured RTECs from LC3+/+ and LC3−/− mice incubated in the absence (control) or presence of TGF-β1 (1 ng/ml) as indicated for 24 hours. Immunoblotting for β-actin was used as the protein loading control. The relative abundance of mature TGF-β was quantitated as the ratio to β-actin by densitometry, and data are presented as the mean±SEM of three independent experiments. *P<0.05 versus LC3+/+ RTECs treated with TGF-β1. (B) Levels of pro–TGF-β and mature TGF-β in HK-2 cells incubated in the absence or presence of TGF-β1 (2 ng/ml) and Baf (10 nM) as indicated for 24 hours. Immunoblotting for β-actin was used as the protein loading control. The relative abundance of mature TGF-β was quantitated as the ratio to β-actin by densitometry, and data are presented as the mean±SEM of three independent experiments. *P<0.05 versus no treatment; **P<0.05 versus TGF-β1 treatment only. (C) Semi-quantitative RT-PCR for TGF-β1 mRNA expression in HK-2 cells incubated in the absence or presence of TGF-β1 (2 ng/ml) and Baf (10 nM) as indicated for 24 hours. Amplified cDNA level of TGF-β1 was standardized with that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Densitometric analysis data are presented as the mean±SEM of three independent experiments. *P<0.05 versus no treatment. (D) Representative confocal photomicrographs of immunofluorescence labeling for LC3 (red) and TGF-β1 (green) in HK-2 cells incubated in the absence or presence of TGF-β1 (2 ng/ml) and Baf (10 nM) as indicated for 24 hours. (E) Effects of conditioned media (CM) from untreated (control) or TGF-β1–treated LC3+/+ and LC3−/− RTECs on Col-I expression in NIH3T3 fibroblasts. The relative abundance of Col-I was quantitated as the ratio to β-actin by densitometry, and data are presented as the mean±SEM of three independent experiments. *P<0.05 versus control CM; **P<0.05 versus TGF-β1–treated CM from LC3+/+ RTECs.
that autophagic activity in RTECs is not high constitutively compared with the podocytes but potently induced with injury after UUO. In the obstructed kidneys, our studies show increased abundance of GFP-LC3 puncta formation in tubular epithelial cells and increased expression of Beclin 1, LC3-I, and LC3-II proteins similar to previous report showing up-regulated Beclin 1 and LC3 and increased autophagic vacuoles in obstructed renal tubules using transmission electron microscopy.6 Thus, these findings indicate that kidney injury after UUO potently induces autophagy in the RTECs.

Recent studies implicate dysregulated autophagy in disorders characterized by fibrosis in various tissues, including cardiac fibrosis, liver fibrosis, and idiopathic pulmonary fibrosis.43 We have recently reported a critical role of autophagy in negatively regulating matrix production in glomerular mesangial cells by promoting degradation of intracellular Col-I induced by TGF-β1.28 Moreover, inhibition of autophagy by a selective chemical inhibitor, 3-methyladenine, has been shown to enhance interstitial fibrosis in the obstructed kidneys after UUO in rats.44 Here, we use a genetic approach to inhibit autophagy and show that deletion of the LC3 gene in mice results in increased collagen expression and deposition in the tubulointerstitial areas of kidneys after injury induced by UUO, suggesting that LC3 may function to inhibit kidney fibrosis.

Given that beclin 1 homozygous knock-out mice display embryonic lethality and die at about embryonic day 7.5,24 whereas other autophagy-related gene knockout mice, such as LC3+/− mice, can survive to birth, Beclin 1 likely has additional functions other than its role in the initiation of autophagy. Indeed, Beclin 1 is known to mediate crosstalk between autophagy and apoptosis. Beclin 1, which contains a Bcl-2 homology-3 domain, was initially identified as a Bcl-2–interacting protein and has an antiapoptotic role in certain conditions, such as nutrient deprivation and hypoxia, possibly as a stress-adaptive mechanism.31 Caspase-mediated cleavage of Beclin 1 generates N- and C-terminal fragments, resulting in loss of its proautophagic activity and, sensitizes cells to apoptotic signals.45 The depletion of Beclin 1 has been shown to trigger caspase-dependent programmed cell death in Caenorhabditis elegans.31 In the UUO model, both induction of autophagy and apoptosis have been shown to occur in a time-dependent manner. Indeed, increased cell death because of apoptosis leading to tubular epithelial loss is a prominent feature in UUO, and our findings show that inhibition of Beclin 1 through heterozygous deletion of beclin 1 gene enhanced RTEC apoptosis in the obstructed kidneys. These data suggest that Beclin 1 functions to protect RTECs from apoptosis after UUO injury.

A hallmark of CKDs is tubulointerstitial fibrosis with excessive matrix deposition produced by myofibroblasts,46 and tubular epithelial cells have been thought to have an active role in this process by EMT and directly contribute to the myofibroblast pool.47–50 However, recent studies of epithelial lineage in models of kidney injury indicate that EMT is unlikely to occur in vivo,5,6 therefore raising the significance of paracrine actions by which tubular epithelial contribution to interstitial fibrosis is through paracrine signaling rather than the EMT. Interestingly, our data from the GFP-LC3 transgenic mice revealed that the RTECs and not the interstitial cells most prominently display autophagic activity, which was evidenced by increased abundance of GFP-LC3 puncta after kidney injury induced by UUO. Furthermore, we found that treatment with exogenous TGF-β1 induces autophagy in primary cultured mouse RTECs and HK-2 cells. Actions of TGF-β1 are well known as potent inducers of ECM production and fibrogenesis, and they are induced during kidney injury, including the actions induced by UUO. Moreover, TGF-β1 can potentiate its actions through autoinduction. Therefore, in the
present study, we focused on investigating the mechanism by which autophagy in tubular epithelial cells regulates ECM proteins produced by interstitial fibroblasts in response to TGF-β1 and kidney injury and fibrosis induced by UUO. We found that the deletion of LC3 resulted in increased mature TGF-β proteins in the obstructed kidneys after UUO. Similarly, blockade of autophagy in RTECs by the use of LC3-deficient RTECs isolated from LC3−/− mice or inhibition of autolysosomal protein degradation by treatment with bafilomycin A1 also resulted in additional increases in mature TGF-β protein levels without corresponding alterations in TGF-β1 mRNA when its expression was induced by exogenous TGF-β1. These data suggest that LC3 functions to decrease mature TGF-β levels in RTECs through autophagic degradation and consequently, reduces TGF-β secretion and suppresses development of interstitial fibrosis induced by UUO.

Evidence for paracrine effects by tubular cell-derived TGF-β1 is corroborated in a transgenic mouse model of conditional overexpression of TGF-β1 in renal tubules, which displayed widespread peritubular deposition of collagen and fibrosis and focal degeneration of nephrons with empty collapsed remnants of tubular basement membrane embedded into a dense collagenous fibrous tissue but no evidence for transition of tubular cells into myofibroblasts. Moreover, in a rat model of ischemia–reperfusion injury, tubular production of TGF-β1 was increased; through paracrine stimulation, in turn, it activated interstitial fibroblasts and led to tubulointerstitial fibrosis, whereas treatment with a pharmacological inhibitor of TGF-β type 1 receptor blocked TGF-β signaling and ameliorated interstitial fibrosis. Our studies show that the conditioned media from TGF-β1–treated LC3-deficient (LC3−/−) RTECs enhanced Col-1 production in NIH3T3 fibroblasts and suggest that LC3 may play a role in regulating mature TGF-β by RTECs and acting in a paracrine fashion to regulate collagen production by adjacent interstitial fibroblasts. Thus, paracrine signaling by tubular epithelial cells through secreted bioactive TGF-β may be an important mechanism that causes interstitial fibrosis.

Regulation of cytokine signaling through autophagic degradation, thereby limiting the availability of the mature cytokine for subsequent processing and secretion, is a novel intracellular mechanism for regulating TGF-β. Interestingly, autophagy recently has been shown to play a role in regulating IL-1 family cytokines. IL-1β is a proinflammatory cytokine that is first produced as a proform, similar to TGF-β, and autophagy controls IL-1β secretion by targeting intracellular pro–IL-1β for degradation. Induction of autophagy occurs in cells stimulated with Toll-like receptor ligands and leads autophagosomes to sequester and degrade pro–IL-1β, damaged mitochondria, and inflammasome components, suggesting that autophagy may regulate inflammation through the degradation. The induction of autophagy in mice using rapamycin reduced LPS-induced elevation of serum IL-1β, and inhibition of autophagy prevented IL-1β degradation and increased mature IL-1β. Moreover, inhibition of autophagy increased inflammasome activation, whereas induction of autophagy repressed it. These studies suggest that the induction of autophagy by inflammatory stimuli may serve as a self-regulatory mechanism by which autophagy controls inflammatory cytokine secretion and reduce active inflammasomes, thereby limiting potentially harmful inflammatory responses. Similarly, our findings suggest a novel role for autophagy as a cytoprotective mechanism to negatively regulate the production of mature TGF-β proteins in RTECs, consequently limiting TGF-β secretion and suppressing development of interstitial fibrosis in kidney injury.

ECM synthesis is important for the initial stage of repair of tubular injury, and transient peritubular myofibroblast differentiation of interstitial fibroblasts may provide beneficial role on tubular recovery as described by Fujigaki et al. Similarly, TGF-β promotes wound repair and regeneration, thereby exerting paradoxical cytoprotective effects to mitigate tissue injury. However, extended dysregulation of expression and activation of TGF-β results in relentless ECM synthesis and accumulation, development of kidney fibrosis, and ultimately, end stage kidney failure. Therefore, the beneficial effects of TGF-β activation and ECM synthesis are transient. Suzuki et al. also reported that tubular injury led to the development of surrounding matrix protein deposition and contributed to the pathogenesis of interstitial fibrosis. Indeed, Fujigaki et al. used a single intravenous injection of uranyl acetate (5 mg/kg) into Sprague–Dawley rats; tubular injury reached its peak 7 days after the injection, and then, tubules underwent a repair and regeneration process. However, our UUO model induces tubular injury consistently without recovery process. Thus, effects of TGF-β activation, ECM synthesis, and activation of interstitial fibroblasts on renal injury may be protective/repairative or deleterious/progressive depending on, for instance, the duration and extent of injury.

In summary, we investigated the functional role of autophagy proteins LC3 and Beclin 1 in kidney injury and fibrosis. The process of autophagy provides a system for protein degradation that is essential for tissue homeostasis and cell survival. We show that the induction of autophagy in RTECs protects against apoptosis and promotes TGF-β degradation, thereby reducing TGF-β secretion and development of renal interstitial fibrosis. Our findings suggest a novel intracellular mechanism for regulating TGF-β by autophagy. Kidney injury, including that induced by UUO, leads to activation of TGF-β, which is a potent inducer of ECM production and fibrogenesis, and can also potentiate its actions through autoinduction. Autophagy may serve as an adaptive mechanism that allows tight control of TGF-β actions.

**CONCISE METHODS**

**Reagents**

Recombinant human TGF-β1 was purchased from R&D Systems (Minneapolis, MN). Bafilomycin A1, anti-LC3 antibody, and...
Cy3-conjugated anti–α-SMA antibody were from Sigma-Aldrich (St. Louis, MO). Anti–Col-1 and anti–TGF-β1 antibodies were from Calbiochem (San Diego, CA) and Abcam (Cambridge, MA), respectively. Antibodies against cleaved caspase-3, caspase-9, and TGF-β were purchased from Cell Signaling Technology (Danvers, MA). Antibodies against Beclin 1 and β-actin were from Santa Cruz Biotechnology (Santa Cruz, CA). Cy3-conjugated anti-rabbit IgG, Cy3-conjugated anti-mouse IgG, and FITC-conjugated anti-rabbit IgG were from Jackson ImmunoResearch Laboratories (West Grove, PA).

Mice and UUO
Male C57BL/6 mice were from The Jackson Laboratory (Bar Harbor, ME). GFP-LC3 transgenic mice were provided by Riken Laboratories (Hirosawa, Japan). LC3+/+ and LC3−/− mice were provided by Marlene Rabinovitch (Standford, CA).60 Beclin 1+/+ and beclin 1−/− mice were provided by Beth Levine (Dallas, TX).61 For UUO, mice (8–12 weeks of age) were anesthetized with pentobarbital, and the left ureter was exposed by a left dorsal incision. The ureter was obstructed by two point ligations with silk sutures. Sham-operated mice underwent the same procedure, except for the obstruction of the left ureter. The incision was closed, and mice were allowed to recover. Mice were euthanized 3, 7, 10, or 14 days after surgery. Kidneys were divided into small pieces (<1 mm) and incubated in HBSS containing 0.2% collagenase II for 1 hour at 37°C in an oxygen-saturated atmosphere. The samples were then passed through sieves of descending pore sizes (250, 150, 68, and 40 μm). The resultant tubular epithelial cell isolates were resuspended and cultured in modified K1 medium containing 5% FCS (Gibco, Egggenstein, Germany), 10 μg/ml EGF, 10 mM Hepes, 0.5 mg/ml prostaglandin E2, 180 μg/ml hydrocortisone, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were used for experiments at passages 3–5 and rendered quiescent in media containing 0.5% FCS for 24 hours before treatment with TGF-β1. Mouse fibroblast (NIH3T3) cells were obtained from American Type Culture Collection and cultured in DMEM with 10% FCS. Supernatants of primary cultured RTECs from Col-I and anti-LC3 as primary antibodies Cy3-conjugated anti-rabbit IgG, Cy3-conjugated anti-mouse IgG, and FITC-conjugated anti-rabbit IgG were used to detect the corresponding primary antibodies. Images were analyzed using a Nikon D-eclipse C1 confocal fluorescence microscope. Exposure settings were unchanged throughout acquisition. Relative area labeled with antibodies to Col-I was assessed in predetermined high-power fields (×200) of the cortex (15 fields), and the percentage of the total tissue area that stained positively for Col-I was determined using ImageJ software (National Institutes of Health, Bethesda, MD).

Western Blot Analysis
Immunoblotting was carried out as previously described.62 Brieﬂy, proteins were extracted with buffer containing 0.05 M Tris–HCl (pH 8.0), 0.15 M NaCl, 5.0 mM EDTA, 1% NP-40, and protease inhibitors [2.0 mM N-ethylmaleimide, 2.0 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 1 μg/ml leupeptin, and pepstatin] at 4°C. After centrifugation (14,000 rpm for 20 minutes), supernatant (detergent-soluble), and pellet (detergent-insoluble) fractions were collected and subjected to Western blotting, and target proteins were detected by using LumiGLO (Cell Signaling Technology, Beverly, MA) and exposed to x-ray films.

RT-PCR
RT-PCR was carried out as previously described.64 The PCR primer sets for human TGF-β1 were (forward) 5′-ACATTGACTTCCGCAAGGAC-3′, (reverse) 5′-GTCCAGGCTCCAAATGTAGG-3′. The PCR primer sets for human glyceraldehyde 3-phosphate dehydrogenase were (forward) 5′-CAATGACCCCTTCATGAG-3′ and (reverse) 5′-TTGATTTTGAGGATCTCG-3′.

TUNEL Assay
TUNEL assay was performed to detect apoptotic cell death using the ApoTag Plus Peroxidase In Situ Apoptosis Detection Kit (Chemicon, Temecula, CA) as described in the manufacturer’s instructions. In each kidney, the numbers of tubular and interstitial apoptotic cells were counted separately in 20 nonoverlapping fields observed at ×400 magnification.

Statistical Analyses
Statistical signiﬁcance of the experimental data from three independent experiments was derived by the t test or ANOVA, and P values<0.05 were considered significant. All of the experiments were performed at least three times. Densiometric analyses for the quantitation of Western blot and RT-PCR data were carried out by using ImageJ software.
ACKNOWLEDGMENTS

We thank Drs. C. Brooks and T. Ichimura (Brigham and Women’s Hospital, Harvard Medical School) for technical assistance.

This work was supported by the National Institutes of Health grants R01-HL079904, P01-HL114501, and R01-DK57661 to M.E.C.

DISCLOSURES

None.

REFERENCES


