Simultaneous deletion of Bax and Bak is required to prevent apoptosis and interstitial fibrosis in obstructive nephropathy

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Jang HS, Padanilam BJ. Simultaneous deletion of Bax and Bak is required to prevent apoptosis and interstitial fibrosis in obstructive nephropathy. Am J Physiol Renal Physiol 309: F540–F550, 2015. First published July 15, 2015; doi:10.1152/ajprenal.00170.2015.—Proximal tubular injury and apoptosis are key mediators of the development of kidney fibrosis, a hallmark of chronic kidney disease. However, the molecular mechanism by which tubular apoptotic cell death leads to kidney fibrosis is poorly understood. In the present study, we tested the roles of Bcl-2-associated X (Bax) and Bcl-2 antagonist/killer (Bak), two crucial proteins involved in intrinsic apoptotic cell death, in the progression of kidney fibrosis. Mice with proximal tubule-specific Bax deletion, systemic deletion of Bak, and dual deletion of Bax and Bak were subjected to unilateral ureteral obstruction (UUO). Dual deficiency of Bax and Bak inhibited tubular apoptosis and atrophy. Consistent with decreased tubular injury, dual ablation of Bax and Bak in the kidney is required to prevent UUO-induced tubular apoptosis and the consequent kidney inflammation and fibrosis.

CHRONIC KIDNEY DISEASE (CKD) remains a life-threatening problem (69). The incidence of CKD along with its main causes, such as diabetes and hypertension, is increasing, resulting in mounting financial burden to the families and societies of the world (1, 64). Kidney fibrosis is a significant characteristic of CKD, leading to the loss of kidney function (64). The cellular mechanisms that lead to renal fibrosis are complex and include inflammation, oxidative stress, and proximal tubule cell apoptosis and cell cycle arrest. Although several molecules, including various cytokines [IL-13, IL-21, and transforming growth factor (TGF)-β1], as well as the renin-angiotensin-aldosterone system have been implicated in renal fibrosis (64), an effective strategy to treat kidney fibrosis remains as a major unmet medical need. Current efforts to optimize renin-angiotensin-aldosterone system blockade and control blood pressure may reduce proteinuria, a surrogate marker of renal disease, but only partially reduce the progression of CKD.

Kidney tubular injury has been recognized to be a primary cause of kidney fibrosis (32, 57, 67). In the kidney, apoptosis is a major cause of tubular cell loss, resulting in tubular atrophy and functional loss (3, 43, 47, 57). Tubular atrophy is associated with the progression of CKD, including kidney fibrosis (57). Reversible injury results in a normal repair process by intrinsic cellular mechanism and/or paracrine effect of extrarenal sources, whereas irreversible injury of tubular epithelial cell, such as tubular atrophy, triggers fibrogenic signaling (4). An abnormal repair process in injured tubular cell has been implicated in G2/M cell cycle arrest, resulting in the secretion of profibrotic cytokines by the arrested cell and the activation of a profibrotic signaling pathway (67). In tubular injury-mediated fibrosis, the inflammatory response is a critical step that is required for the initiation and progression of kidney fibrosis. Activation of the innate immune system by injured epithelial cell-secreted profibrotic cytokines, such as IL-1, IL-6, and TNF-α, not only trigger kidney inflammation but also result in fibroblast activation (66). A number of studies have demonstrated that blockage of profibrotic cytokines or deletion of immune cells, including macrophages, could prevent kidney fibrosis (9, 66).

Bcl-2-associated X (Bax) and Bcl-2 antagonist/killer (Bak) are proapoptotic members of the Bcl-2 family that govern mitochondrial outer membrane permeabilization to elicit apoptotic cell death (62). Several reports have demonstrated that proapoptotic proteins, including Bax and Bak, are associated with apoptotic and necrotic cell death in kidney diseases and that blockade of their functions could blunt disease progression (8, 36, 43, 63, 70). However, a direct role of Bax and Bak in kidney fibrosis, in isolation or in combination, has not been studied. In the present study, we investigated the role of Bax and Bak in kidney fibrosis and whether dual inhibition of their functions synergistically prevents unilateral ureteral obstruction (UUO)-induced tubular apoptosis and interstitial fibrosis.

MATERIALS AND METHODS

Mice and surgical preparation. Mice were cared for and during the experimental procedures in accordance with the policies of the Institutional Animal Care and Use Committee of the University of Nebraska Medical Center and the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. All protocols received prior approval from the Institutional Animal Care and Use Committee of the University of Nebraska Medical Center. Bax+/− and Bak+/− mice (52) (Jackson Lab, Bar Harbor, ME) were crossed to generate Bax+/−Bak−/− mice. These mice were then bred with Pepck Cre recombinase-expressing mice (a kind gift from Dr. Volker Haase, Vanderbilt University) to generate mice with proximal tubule-specific knockout (KO) of Bax and Bak (Bax-null and Bak-mice). C57BL/6J, Bax+/− with Pepck Cre recombinase, Bak-null, and double-KO Bax and Bak mice were anesthetized by intraperitoneal injection of a cocktail containing ketamine (200 mg/kg body wt) and xylazine (16 mg/kg body wt). The right ureter was obstructed completely near the...
renal pelvis using 5-0 silk, as previously described (19). Sham-operated mice underwent the same surgical procedure except for the ureter ligation. After 7 days of UUO or sham surgery, kidneys were either fixed in 4% formaldehyde for histological experiments or snap frozen in liquid nitrogen for biochemical experiments. Periodic acid-Schiff-stained sections were used to determine histological damage scores as previously described (17).

Collagen deposition. Collagen deposition was assessed by Sirius red staining as previously described (28). Sirius red-positive areas were expressed as the ratio of the Sirius red-positive area to total area in five randomly chosen fields per kidney.

Histology and evaluation of tubular injury. Periodic acid-Schiff-stained sections were used to evaluate tubular injury. Tubular atrophy and dilatation were expressed as the ratio of numbers of atrophied or dilated tubules to numbers of total tubules in five randomly chosen fields per kidney.

Apoptotic cell death. A TUNEL assay on kidney sections to evaluate apoptotic cells was carried out using the In Situ Cell Death Detection kit with fluorescein (Roche, Mannheim, Germany) as previously described (16).

Immunohistochemistry and immunofluorescent staining. Immunohistochemical staining of the kidneys was performed on paraffin-embedded sections as previously described (18, 21). Briefly, 4% paraformaldehyde-fixed kidney sections were rehydrated and labeled with antibodies against α-smooth muscle actin (α-SMA; Sigma, St. Louis, MO), polymorphonuclear neutrophil (PMN; Accurate, Westbury, NY), F4/80 (Proteintech, Chicago, IL), phosphorylated (p)-histone H3 (Santa Cruz Biotechnology, Santa Cruz, CA), p-Smad3 (Abcam, Cambridge, MA), and Ki67 (Novus Biologicals, Littleton, CO). Sections were then incubated with peroxidase- or FITC-conjugated secondary antibodies (Vector Laboratories, Burlingame, CA). 4,6-Diamidino-2-phenylindole dihydrochloride (Sigma) or Meyer’s hematoxylin (Electron Microscopy Sciences, Hatfield, PA) were used to stain nuclei. The α-SMA-positive area was measured in five randomly chosen fields per kidney using ImageJ software (NIH, Baltimore, MD). Respective numbers of PMN+, F4/80+, p-histone H3+, and Ki67-positive cells were counted in five randomly chosen fields per kidney.

Western blot analysis. Western blot analysis was conducted as previously described (20) using various antibodies against the following proteins: connective tissue growth factor (CTGF), TGF-β1, ICAM-1, p53, and GAPDH (Santa Cruz Biotechnology), p-EGF receptor (EGFR), p-Smad3 and p-JNK (Cell Signaling), fibronectin (Cedarlane, Burlington, NC), α-SMA (Sigma), TNF-α, IL-1β, and IL-6 (Abcam), and cyclin B1, cyclin D1, and cleaved caspase-3 (Cell Signaling). GAPDH immunoblot analysis was used as a loading control on stripped membranes. Band intensities were analyzed by ImageJ software (NIH).

Measurement of lipid peroxidation. The level of lipid hydroperoxide in the kidneys was measured using a lipid hydroperoxide assay kit (Cayman) as previously described (28).

Statistical analyses. ANOVA was used to compare data among groups. Differences between two groups were assessed by two-tailed Student’s t test.

Figure 1. Inhibition of unilateral ureteral obstruction (UUO)-induced kidney tubular apoptosis by dual ablation of Bax and Bak. Four groups of mice [wild-type (WT), Bcl-2-associated X (Bax)−/−;Pepck-Cre [Bax proximal tubule (PT)-specific knockout (KO)], Bcl-2 antagonist/killer (Bak)-null (Bak KO), and Bax−/−;Pepck Cre; Bak-null [double KO (DKO)] mice] were subjected to UUO or sham operation for 7 days. A: tubular apoptosis was evaluated by TUNEL assay. B: TUNEL-positive cells were measured in five randomly chosen fields per kidney. C: expression of cleaved caspase-3 was examined by Western blot analysis. Anti-GAPDH antibody was used as a loading control. D: Western blot band densities were evaluated using ImageJ software. Green fluorescence indicates TUNEL-positive cells. Arrow, tubular TUNEL-positive cells. CLK, contralateral kidney. Data are expressed as means ± SE; n = 3, *P < 0.05 vs. respective sham-operated mice; #P < 0.05 vs. UUO in WT mice.
Student’s t-test. *P values of <0.05 were considered statistically significant.

RESULTS

Effect of deletion of Bax and Bak in tubular apoptosis and atrophy after UUO. To investigate the role of the proapoptotic proteins Bax and Bak in UUO-induced kidney fibrosis, we generated mice with proximal tubule-specific deletion of Bax and/or Bak. Mice were subjected to UUO, a well-established kidney fibrosis model, for the induction of kidney fibrosis. As in our previous studies (19, 21, 24, 26), UUO induced severe tubular cell damage and death, including tubular dilatation, atrophy, apoptosis, and necrosis in the kidney (Figs. 1A and 2A). Dual deletion of Bax and Bak resulted in less TUNEL-positive apoptotic tubular epithelial cell death and expression of cleaved caspase-3 in the kidney during UUO compared with contralateral kidneys derived from wild-type and single deletion mutants of Bax or Bak (Fig. 1). Bax or Bak KO mice showed a decrease in apoptotic cell death in UUO-kidneys, but this was not significantly different compared with that in WT kidneys (Fig. 1). Similar to apoptotic cell death, dual ablation of Bax and Bak showed a decrease in atrophied tubules (Fig. 2B). Tubular necrosis, including dilatation, was similar in all groups after UUO (Fig. 2C). These results indicate that dual ablation of Bax and Bak is required to prevent UUO-induced apoptotic cell death and the subsequent tubular atrophy.

Dual loss of Bax and Bak inhibits UUO-induced kidney inflammation. To determine the relationship between apoptotic cell death and consequent atrophy as well as the inflammation level in UUO kidneys, we examined neutrophil and macrophage recruitment into UUO kidneys. Infiltration of cells into the kidney was evaluated by counting the numbers of PMN-positive cells for neutrophils and F4/80-positive cells for macrophages. Numbers of both neutrophils and macrophages were significantly increased in the kidneys after UUO (Fig. 3). Bax or Bak ablation did not result in significant differences in the number of inflammatory cells compared with those of WT kidneys (Fig. 3). However, unlike Bax or Bak single deletion, dual ablation of Bax and Bak markedly blocked neutrophil and macrophage infiltration into UUO kidneys (Fig. 3).

The inflammatory response was further examined by analyzing the expression of ICAM-1, a critical factor involved in the recruitment of leukocytes to injured tissue. The expression level of ICAM-1 increased proportionally to the number of neutrophils and macrophages recruited into UUO kidneys (Fig. 4, A and B). Proinflammatory cytokines (IL-6, TNF-α, and IL-1β), which could be secreted by inflammatory immune and injured tubule cells, was also upregulated in UUO kidneys of WT and Bax or Bak single deletion mice but suppressed in those of double-KO mouse kidneys (Fig. 4, A and C–E). These data suggest that lowered tubular apoptotic cell death and atrophy observed in double-KO mouse kidneys after UUO may lead to decreased inflammation levels.

Next, to determine whether an attenuated oxidative stress level is involved in preventing the injury in dual KO of Bax and Bak, oxidative stress levels were analyzed in the different mouse groups. In line with our previous studies, UUO significantly elevated oxidative stress levels, as determined by lipid peroxidation levels (Fig. 4F). Dual KO of Bax and Bak reduced UUO-induced oxidative stress in the kidney compared with those of WT, Bax KO, or Bak KO kidneys (Fig. 4F). These data suggest that double deletion of Bax and Bak...
prevents increased oxidative stress from inducing kidney fibrosis, but single deletion of Bax may be compensated by Bak or vice versa and is ineffective in protecting the kidneys. Dual deficiency of Bax and Bak alleviates UUO-induced tubular cell cycle arrest. Proximal tubule injury in UUO kidneys is associated with loss of tubular function and tubular cell cycle arrest leading to kidney fibrosis (67). To evaluate cell cycle arrest as a function of Bax and Bak, we evaluated the number of p-histone H3-positive cells, a marker for the G2/M phase, in UUO kidneys. The number of p-histone H3-positive cells was significantly increased in WT kidneys with UUO compared with sham-operated control mouse kidneys (Fig. 5, A and B). The ratio of cyclin B1 to cyclin D1, a marker of G2/M arrest, showed a similar pattern with expression of p-histone H3 (Fig. 5, C and D). However, dual deletion of Bax and Bak prevented cell cycle arrest, as shown by fewer p-histone H3-positive cells and a decreased ratio of cyclin B1 to cyclin D1 in the kidney compared with all other groups after UUO (Fig. 5, A–D). Bax or Bak single deletion did not result in decreases of p-histone H3-positive cells and the ratio of cyclin B1 to cyclin D1 compared with those of WT kidneys (Fig. 5, A–D). Furthermore, to define the relationship between Bax and Bak as well as cell cycle arrest-related factors in UUO kidneys, we examined the level of p53 in UUO kidneys. It is known that p53, the earliest responder of DNA damage, is a key molecule regulating the cell cycle, including cell cycle arrest, as well as apoptosis (10, 33). UUO kidneys revealed an upregulation of p53 compared with control kidneys (Fig. 5, C and E). In line with G2/M arrest, the expression level of p53 decreased in dual Bax and Bak-deleted kidneys but not in Bax or Bak single-deleted UUO kidneys (Fig. 5, C and E). These data suggest that dual deletion of Bax and Bak may prevent UUO-induced tubular cell cycle arrest by regulating p53 expression.

Deletion of Bax and Bak inhibits UUO-induced kidney fibrosis. To determine whether dual ablation of Bax and Bak and the consequential decreased tubular apoptosis and inflammation indeed prevent kidney fibrosis after UUO, we evaluated the levels of extracellular matrix protein (ECM) deposition and fibrotic markers. UUO kidneys showed a significant increase of collagen deposition, as measured by Sirius red-positive areas (Fig. 6, A and B). Similarly, another ECM protein, fibronectin, was markedly enhanced in the kidney after UUO (Fig. 6, E and F). Expression of α-SMA, a marker of fibroblast activation and myofibroblast formation, was markedly increased (Fig. 6, C and D). Dual ablation of Bax and Bak significantly inhibited UUO-induced collagen and fibronectin deposition as well as...
the expression of α-SMA, whereas expression of these proteins in Bax or Bak KO mice did not differ from those of WT mice (Fig. 6). These data demonstrate that the reduced apoptotic cell death and inflammation in Bax- and Bak-deficient mice attenuated the expression of ECM and fibrotic proteins, resulting in inhibition of kidney fibrosis.

**Double KO of Bax and Bak reduces fibrogenic signaling and interstitial cell proliferation in the UUO kidney.** To delve into the molecular mechanism by which dual deletion of Bax and Bak prevents kidney fibrosis after UUO, we analyzed the expression and activation of key fibrogenic signals that may trigger kidney fibrosis. Since it has been recognized that activation of EGFR, Smad3, and JNK is associated with TGF-β-dependent kidney fibrosis (5, 34, 45, 67), we first examined the expression of these molecules in the mouse kidney after UUO. Expression levels of all these proteins were upregulated in UUO WT kidneys but were suppressed in dual-KO Bax and Bak kidneys (Fig. 7, A–D). Increased expression of TGF-β, a downstream signal of EGFR, coincided with the increased fibrosis in UUO kidneys. Dual ablation of Bax and Bak prevented the augmentation of TGF-β compared with other groups (Fig. 7, A and E). CTGF, which triggers kidney fibrosis in a TGF-β-independent manner (44), was also increased in UUO kidneys, but its expression level was attenuated in dual-KO Bax and Bak mice (Fig. 7, A and F). In line with the results from Western blot analysis, immunohistochemistry for p-Smad3 showed that UUO elevated the number of p-Smad3-positive cells in WT kidneys but not those of double-KO Bax and Bak kidneys (Fig. 7G). These findings indicate that dual KO of Bax and Bak may attenuate fibrosis by blocking both TGF-β-dependent and -independent mechanisms. Next, we determined the effect of dual KO of Bax and Bak in interstitial and tubular cell proliferation. UUO increased the proliferation of interstitial cells (Fig. 8, A–B), which include resident fibroblasts, bone marrow-derived cells, and pericytes (31). Interstitial cell proliferation was suppressed in kidneys of mice with dual deletion of Bax and Bak compared with those of WT, Bak KO, and Bak KO kidneys after UUO (Fig. 8B). Tubular cell proliferation was not altered between WT or single-KO or dual-KO UUO kidneys (Fig. 8C). These results demonstrate that dual deficiency of Bax and Bak inhibits kidney fibrosis by suppressing fibrogenic signaling and interstitial cell proliferation.

**DISCUSSION**

The major forms of cell death in the injured tubule are apoptosis and necrosis (3, 43). During the past several decades, tubular apoptosis has been established as an important contributor to kidney fibrosis (3, 57). Although apoptosis was initially considered to be inconsequential to the development of fibrogenesis, a recent report (23) has suggested that apoptosis-related proteins are directly related with disease progression. However, the molecular mechanisms by which proapoptotic proteins and apoptotic cells may trigger fibrogenesis have not been established.

In the present study, we defined the role of apoptosis in kidney fibrosis using mice with proximal tubule-targeted dele-
tion of Bax and systemic deletion of Bak. We showed that prevention of tubular apoptosis by dual inhibition of Bax and Bak resulted in the suppression of kidney fibrosis and inflammation in the UUO model of CKD (Fig. 9). This protective effect seems to be associated with inhibition of apoptosis-induced tubular injury and inflammation as well as cell cycle arrest. Intriguingly, single KO of Bax or Bak did not result in the prevention of apoptosis or kidney fibrosis, suggesting there is a synergy between Bax and Bak in inducing apoptotic cell death and that loss of one may be compensated by the other. Our results agree with a previous report (60) showing that mouse embryonic fibroblasts with dual deletion of Bax and Bak, but not Bax or Bak, are resistant to apoptosis when exposed to drugs inducing mitochondrial dysfunction but not to extrinsic death signals such as TNF-α plus actinomycin D. Moreover, global double KO of Bax and Bak showed less hepatocyte apoptosis and delayed mortality in mice administered agonistic antibody to Fas, by preserving mitochondrial function (60).

Proximal tubule injury plays a major role in the initiation and progression of kidney fibrosis (4, 14, 41). Proximal tubule cell recovers completely or incompletely after injury, depending on the injury burden and duration (12, 67). Injured proximal tubule cells that fail to fully recover, due to irreversible damage, can trigger the release or activation of fibrogenic and inflammatory signals (4, 57). Recent reports (12, 48) have demonstrated that selective tubular cell injury, using transgenic mice with diphtheria toxin receptor, resulted in chronic kidney injury, including kidney fibrosis and inflammation. Gracic et al. (12) showed that single injection of diphtheria toxin allowed the injured kidney to fully recover but that repetitive administration led to kidney fibrosis, indicating that irreversible and sustained tubular damage can induce kidney fibrosis.

Tightly regulated apoptosis is beneficial in normal and mildly injured cells and organs to preserve their functions (13, 23). Moreover, proper apoptosis in the kidney could prevent tubular necrosis, thereby suppressing inflammation and subsequent fibrosis (13). However, dysregulated and excessive apoptosis could result in functional loss, leading to chronic kidney failure (25, 39, 40, 51, 57). Although it is possible that Bax/Bak may also contribute to anti-inflammatory pathways, such signaling pathways remain to be defined. In the kidney, prolonged and severe injury induce massive tubular apoptosis and subsequent tubular atrophy (47, 57). Failure of adjacent epithelial cells to replace the denuded region by dedifferentiation and proliferation leads to functional loss of the kidney (3, 57). Apoptotic cells could stimulate fibrogenesis by both direct and indirect pathways (23). Damaged tubule-secreted fibro-
BAX AND BAK IN KIDNEY FIBROSIS

Fig. 6. Ablations of both Bax and Bak prevent kidney fibrosis. Mice were subjected to UUO or sham operation for 7 days. A and C: collagen deposition (A) and α-smooth muscle actin (α-SMA)-positive areas (C) were evaluated by Sirius red and immunofluorescent staining with anti-α-SMA antibody in paraffin-embedded kidneys, respectively. B and D: Sirius red-positive (B) and α-SMA-positive (D) areas were measured in five randomly chosen fields per kidney using ImageJ software. E: fibronectin expression was examined by Western blot analysis. Anti-GAPDH antibody was used as a loading control. F: Western blot band densities were evaluated using ImageJ software. Data are expressed as means ± SE; n = 3. *P < 0.05 vs. respective sham-operated mice; #P < 0.05 vs. UUO in WT mice.

genic factors, such as TGF-β and CTGF, trigger the activation of fibroblasts and recruitment of immune cells into the damaged site. A recent report (30) has suggested that apoptotic endothelial cells generate CTGF production, leading to skin fibrosis. The apoptotic cell body is phagocytosed to resolve inflammation by macrophages, but it secretes profibrotic cytokines and growth factors to stimulate tissue restoration, ultimately leading to tissue fibrosis (37, 42, 50). Failed phagocytosis of the apoptotic cell body by macrophages could directly result in tissue inflammation and fibrosis (11). Indeed, Wang et al. (58) demonstrated that administered apoptotic cells labeled with fluorescent beads into the lung were phagocytosed by macrophages but still remained in the lung during several weeks and elicited lung inflammation and fibrosis via upregulation of TNF-α and TGF-β, showing direct evidence of tissue inflammation by massive apoptotic cell bodies. Furthermore, inactivation of caspase-3, which is a final cascade of both death receptor- and mitochondria-mediated apoptosis, prevents non-alcoholic steatohepatitis-associated liver fibrosis through suppression of the inflammatory response by inhibition of apoptotic cell death (55). Particularly, several cytokines seems critical factors to progress tissue fibrosis. It has been reported that IL-6...
levels of cell cycle regulator proteins, p53 and p21, and G2/M detection of cell cycle arrest in kidney fibrosis: of these, the formation (2, 22, 65). Several markers have been used for the association with the progression of kidney diseases and could be suppress its progression, although their roles are cell dependent

promote cell cycle progression, whereas antiapoptotic factors progression in tumor cells. In general, proapoptotic factors signals to prevent kidney fibrosis.

inflammation and inhibition of cell cycle arrest and fibrogenic signals to prevent kidney fibrosis.

It is well known that Bcl-2 family genes regulate cell cycle progression in tumor cells. In general, proapoptotic factors promote cell cycle progression, whereas antiapoptotic factors suppress its progression, although their roles are cell dependent (52, 71). Tubular epithelial cell cycle arrest is a critical factor associated with the progression of kidney diseases and could be triggered by both acute and chronic kidney injury and inflammation (2, 22, 65). Several markers have been used for the detection of cell cycle arrest in kidney fibrosis: of these, the levels of cell cycle regulator proteins, p53 and p21, and G2/M checkpoint proteins, p-histone H3 and checkpoint kinase, have been investigated for the identification of cell cycle-arrested cells (15, 27, 38, 68). The data presented in this study show that p-histone H3 and the ratio of cyclin B1 to cyclin D1, a marker of G2/M phase and arrest, are increased in tubular epithelial cells of UUO kidneys. Furthermore, the expression of p53 protein, which is involved in cell cycle arrest, is upregulated (56). Deletion of Bax and Bak prevented the increase in the expression of the aforementioned cell cycle proteins, suggesting that inhibition of tubular apoptosis and the consequent decreased inflammation and oxidative stress could prevent cell cycle arrest in kidney fibrosis.

Activation of JNK is another mechanism that can elicit epithelial cell cycle arrest, inflammation, and fibrosis (6, 29, 54, 67). Pharmacological inhibition of JNK blocks the secretion of fibrogenic molecules in kidney tubular cells with G2/M arrest (67). Tubular JNK activation has also been correlated with the expression of inflammatory cytokines in TGF-β- and IL-1β-treated rat tubular cells, ischemia-reperfusion injury-induced rat kidney fibrosis, and in kidney tubular epithelial cells in patients with diverse kidney diseases (6). Moreover, activation of JNK stimulates fibrogenic signals, such as TGF-β and CTGF (29). These reports support our current findings that Bax and Bak deficiency-mediated decreases of JNK activation may lead to the prevention of cell cycle arrest and inflammatory molecules, including TNF-α, IL-6, and IL-1β.

EGFR can trigger kidney fibrosis in CKD models of ischemia-reperfusion injury, UUO, and ANG II infusion (5, 34, 53). Chen et al. (5) reported that chronic ANG II infusion activates EGFR-mediated fibrogenic signaling, resulting kidney fibrosis. Consistent with these data, sustained EGFR activation in the kidney leads to tubular cell cycle arrest and tubular apoptosis, resulting in kidney fibrosis (53). Genetic or pharmacological intervention of EGFR prevented UUO-induced kidney fibrosis (34). EGFR signaling can activate TGF-β/Smad3 signaling, a well-established driving force for kidney fibrosis that potentiates fibroblast activation and ECM secretion from tubular and interstitial cells (35, 44). Furthermore, Yang and colleagues (67) showed that TGF-β is elevated in in vivo and in vitro kidney tubular cell with G2/M arrest. Our present data reveal that EGFR activation is suppressed in UUO kidneys with dual KO of Bax and Bak, along with downregulation of TGF-β. CTGF can trigger kidney fibrosis in a TGF-β-independent pathway as well as in cooperation with TGF-β
We observed that CTGF also showed the same pattern as that of TGF-β in UUO kidneys with dual KO of Bax and Bak. Samarakoon et al. (46) reported that TGF-β can reciprocally activate EGFR resulting in the expression of profibrogenic genes, such as CTGF. Collectively, these results suggest that Bax/Bak deletion may indirectly regulate the EGFR-TGF-β-CTGF axis to induce kidney fibrogenesis.

A previous report (49) has suggested that Bax or Bak KO is sufficient to prevent apoptotic cell death in in vitro and in vivo models of injury. Wei and colleagues (61) reported that single KO of Bak or Bax protects the kidney from ischemia-reperfusion injury via inhibition of tubular apoptosis and mitochondrial fragmentation. However, mice with dual deletion of Bax and Bak in proximal tubules have not been studied in any disease models. Our current results, for the first time, show that deletion of both Bax and Bak is required to prevent kidney fibrosis through the suppression of tubular apoptotic cell death and the subsequent inflammation and cell cycle arrest. The discrepancy in apoptosis may be caused by a distinct mechanism between experimental models of severe ischemia-reperfusion injury versus UUO.

Collectively, our findings suggest that tubular epithelial apoptosis and the subsequent downstream activation of inflammatory signaling and oxidative stress can induce kidney fibrosis. Although Bax and Bak deletion did not prevent necrotic cell death in kidney fibrosis, our data show that their combined deletion prevents kidney fibrosis via the suppression of apoptosis, inflammatory, and fibrogenic signaling. The development of pharmacological agents targeting both Bax and Bak is required to prevent apoptosis, which might represent a novel strategy for the treatment of kidney fibrosis or slowing its progression.

Fig. 9. A scheme for the role of Bax/Bak in UUO-induced kidney fibrosis. PTC, proximal tubule cells; ECM, extracellular matrix.

Fig. 8. Dual ablation of Bax and Bak reduces UUO-induced interstitial cell proliferation in the kidney. Mice were subjected to UUO or sham operation for 7 days. A: paraffin-embedded kidney sections were used for immunofluorescent staining with anti-Ki67 antibody. B and C: interstitial (B) and tubular (C) Ki67-positive cells were measured in five randomly chosen fields per kidney. Arrow, interstitial Ki67-positive cells. Data are expressed as means ± SE; n = 3. *P < 0.05 vs. respective sham-operated mice; #P < 0.05 vs. UUO in WT mice.
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