Mechanisms of Renal Apoptosis in Health and Disease

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ABSTRACT
Apoptotic cell death is usually a response to the cell microenvironment. Apoptosis requires the activation of lethal molecules and the inactivation of prosurvival ones. Both are potential therapeutic targets. Apoptosis contributes to parenchymal cell loss in the course of acute and chronic renal injury. Apoptotic pathways that are active in glomerular and tubular epithelium include death induced by survival factor deprivation, death receptor activation, mitochondrial injury, endoplasmic reticulum stress, lysosomal destabilization, and caspase cascade activation. These pathways are not mutually exclusive, and stimulus-specific differences in the recruitment of apoptotic pathways have been observed. In some cases, the activation of a certain death pathway is redundant, and its inhibition does not prevent eventual cell death. This review summarizes recent advances in the field and discusses the rational basis to choose from the available tools to target apoptosis therapeutically.

OVERVIEW OF APOPTOSIS PATHWAYS

Cell death is usually a response to the cell microenvironment. Maintenance of cell survival is a consequence of the interplay between the presence of survival factors that activate intracellular survival pathways and keep the lethal pathways dormant and the presence of lethal factors that activate the latter and lead to apoptosis (Figure 1). Surrounding cells, soluble mediators, nutritional factors, and the extracellular matrix regulate cell survival. Surrounding cells also express survival or lethal factors or compete for such factors. Two main intracellular pathways for apoptosis have been recognized (Figure 2): Ligation of plasma membrane death receptors (extrinsic pathway) and perturbation of the intracellular homeostasis (intrinsic pathway).

In the extrinsic pathway ligation of death receptors leads to the assembly of multimolecular complexes that include and chronic kidney diseases. Examples are podocytopenia and tubular cell loss in acute kidney injury (AKI) and chronic tubular atrophy. Apoptosis of renal cells may also be beneficial and help fine-tune the number of renal cells created during recovery from injury by balancing an exaggerated proliferative response. The characterization of the molecular pathways activated at each stage and an understanding of the time frames will be crucial to developing sensible therapeutic strategies. Although lethal factors result in tissue injury, it is commonly thought that competition for survival factors is a key determinant of survival during recovery.

Apoptosis is differentiated from necrosis by morphologic and functional features and by the requirement for energy and intracellular proapoptotic proteins. The relative contribution of apoptosis and necrosis to injury is variable and depends on the severity of the insult. More severe insults or absent sources of cell energy will result in necrosis. In addition, apoptotic cells that are not engulfed by phagocytes or epithelia will undergo secondary necrosis.
Lethal factors
Apaf-1
Bad
Bax
Cyclin I
BclxL/Bcl2

Apart from the lack of survival factors or the presence of lethal factors may induce apoptotic cell death. Among lethal factors, lethal cytokines activate the extrinsic pathways for apoptosis by binding to death receptors containing a death domain. In addition, different types of cell stress or cytokines not binding death receptors may activate the intrinsic pathway for apoptosis. Mitochondria are key elements of the intrinsic pathway; however, ER or nuclear or lysosomal injury may also promote apoptosis. The cleavage and activation of Bid by caspase-8 links the extrinsic and intrinsic pathways.

**Figure 1.** Extracellular survival and lethal factors regulate cell susceptibility to apoptosis. Either the lack of survival factors or the presence of lethal factors may induce apoptotic cell death. Among lethal factors, lethal cytokines activate the extrinsic pathways for apoptosis by binding to death receptors containing a death domain. In addition, different types of cell stress or cytokines not binding death receptors may activate the intrinsic pathway for apoptosis. Mitochondria are key elements of the intrinsic pathway; however, ER or nuclear or lysosomal injury may also promote apoptosis. The cleavage and activation of Bid by caspase-8 links the extrinsic and intrinsic pathways.

**Figure 2.** Main intracellular proteins regulating apoptotic cell death. DD, death domain; DED, death effector domain.

Adaptor proteins such as FADD and the activator caspase-8 and -10. These caspases are activated upon oligomerization and then cleave protein substrates to activate downstream effector caspases. The intrinsic pathway involves intracellular organelles, the most important being mitochondria. Sentinel activator BH3-only proteins trigger the allosteric activation of Bax and/or Bak, which oligomerize at the mitochondria, inducing permeabilization of the outer mitochondrial membrane and releasing proapoptotic factors such as cytochrome c, SMAC/DIABLO, and apoptosis-inducing factor (AIF), which promote caspase-dependent and -independent apoptosis. Cytochrome c facilitates the oligomerization of Apaf-1 and caspase-9 in the apoptosome, resulting in activation of caspase 9. Caspase-9 cleaves and activates effector caspases such as caspase-3 and -7, resulting in widespread proteolysis and commitment to cell death.

Signaling cross-talk exists between the intrinsic and extrinsic pathways. The proapoptotic BH3-only protein Bid can be cleaved and activated by caspase-8. The active fragment tBid subsequently translocates to mitochondria and recruits the intrinsic pathway.

Bcl2-related antiapoptotic proteins, such as Bcl2 and BclXL, bind and sequester activator BH3-only proteins, Bax, and Bak and may directly inhibit Apaf-1-mediated activation of caspase-9. Sensitizer BH3-only molecules competitively displace activator BH3-only proteins from the Bcl2 pocket. Inhibitor of apoptosis proteins (IAP) directly inhibit caspases and are, in turn, inhibited by SMAC/DIABLO. Part of the cytoprotective effects of heat-shock protein 27 and heat-shock protein 70 is related to inhibition of key mediators of apoptosis.

Within this general scheme of apoptosis regulation are stimulus-specific pathways. We review apoptosis pathways activated by clinically relevant stimuli. In addition, there are cell-specific pathways. As an example, cyclin I and nephrin are podocyte proteins with antiapoptotic activity (Figure 3).}

**Figure 3.** Cell type-specific apoptosis pathways: Podocyte proteins promote cell survival by activating the AKT pathways, increasing BclXL and Bcl2 expression, and decreasing Bax expression. Includes information from Brinkkoetter et al.16

**Promoting Cell Survival**
Survival factors for tubular cells and podocytes such as hepatocyte growth
factor, erythropoietin, vascular endothelial growth factor, IGF-1, EGF, and parathyroid hormone-related protein activate phosphatidylinositol-3-kinase that, in turn, phosphorylates and activates AKT.\textsuperscript{20–26} AKT provides survival signals by several independent mechanisms.\textsuperscript{27} AKT directly phosphorylates and inhibits proapoptotic factors such as BAD and others. Unphosphorylated BAD binds to Bcl-xL, blocking its survival function. BAD dephosphorylation is observed in tubular cells exposed to proapoptotic stimuli.\textsuperscript{22,23} In addition, phosphorylation by AKT limits the nuclear translocation of the Forkhead family of proapoptotic factors and promotes antiapoptotic NF-κB and Mdm2, an antagonist of p53.\textsuperscript{27} These or similar mechanisms may account for the development of a general intracellular milieu favoring survival in tubular cells exposed to survival factors. This milieu includes low levels of proapoptotic Fas receptor and Bax and higher levels of antiapoptotic Bcl-xL and Bcl2.\textsuperscript{28,29} The situation is reversed in the absence of survival factors.

Regenerative recovery from AKI is associated with activation of survival pathways. AKT engages in tubular cells after renal ischemia/reperfusion injury; tubular cell Bcl-xL increases in experimental AKI, and abrogation of endogenous vascular endothelial growth factor in cyclosporin A (CsA) nephrotoxicity increases renal injury.\textsuperscript{24,28,30} These mechanisms are potentiated by therapeutic intervention. IGF-1, hepatocyte growth factor, erythropoietin, and nonerythropoietic erythropoietin-like molecules prevent experimental AKI, decreasing apoptosis and improving renal function\textsuperscript{20,21,31–33}; however, in randomized, controlled trials, IGF-I failed either to accelerate the recovery of renal function in intensive care patients with AKI or to prevent AKI in cadaveric kidney grafts.\textsuperscript{34,35} Ongoing clinical trials are exploring the role of erythropoietin in prevention of AKI after kidney transplantation.\textsuperscript{36} Survivin, another IAP, has recently been identified as a constitutive prosurvival molecule in tubular cells that protects from experimental AKI.\textsuperscript{37,38}

**ENDOGENOUS PROMOTERS OF APOPTOSIS**

The best characterized endogenous promoters of apoptosis are death receptors containing a death domain (DD).\textsuperscript{11,12} They include receptors for TNF-α, FasL, and TNF-related apoptosis inducing ligand (TRAIL). Cellular inhibitory proteins such as Fas-associated death domain-like interleukin-1beta-converting enzyme (FLICE) inhibitory protein or IAP under most circumstances actively suppress the triggering of apoptosis by death receptors. In this regard, death receptors may activate NF-κB. NF-κB has antiapoptotic activity by inducing Fas-associated death domain-like interleukin-1beta-converting enzyme (FLICE) inhibitory protein, Bcl-xL, c-IAP, and XIAP expression.\textsuperscript{39,40} In the complex environment of the injured kidney, other cytokines may provide predisposing signals for death. In tubular cells, TNF-α-induced apoptosis is facilitated by deprevation of survival factors, whereas FasL requires the upregulation of Fas receptor expression by survival factor deprivation or the presence of an inflammatory milieu, and TRAIL is more lethal in a high-glucose inflammatory milieu.\textsuperscript{28,29,41} The importance of cooperation between lethal factors is underscored by analyses of complex biologic systems.\textsuperscript{42} Changes in the level of expression or activation of apoptosis regulatory molecules may explain the cooperation of cytokines in inducing cell death. As an example, TNF-α increases the expression of TNF-like weak inducer of apoptosis (TWEAK) receptor, Fas, Bax, and Smac/DIABLO while decreasing Bcl-xL in tubular epithelia.\textsuperscript{28,43,44} TWEAK alone does not induce apoptosis in tubular cells; however, in the presence of TNF-α and IFN-γ, the proliferative response results in apoptosis.\textsuperscript{45} TNF-α, FasL, and TWEAK have been successful therapeutic targets in AKI.\textsuperscript{43,46,47} The requirement for cytokine cooperation to induce tubular cell death effectively underlies the observation that targeting individual cytokines has a therapeutic benefit. In addition, members of the TNF superfamily have a broad spectrum of nonlethal activities, including inflammation.

Tubular FADD is upregulated in experimental AKI.\textsuperscript{48} FADD-DD is a truncated molecule corresponding to the DD of FADD that behaves as a FADD antagonist in some cell systems. Surprising, in tubular cells, FADD-DD is sufficient to promote a caspase-independent form of cell death.\textsuperscript{48} This is consistent with a role for FADD in death receptor–independent events, because both FADD-DD and FADD prevent NF-κB activation by toll-like receptors and IL-1β.\textsuperscript{49}

Additional cytokines may induce apoptosis by triggering the intrinsic pathway of apoptosis independent of death receptors. Attention has recently been drawn to the lethal effect of TGF-β1, angiotensin II, and glucose in renal tubular epithelial cells and podocytes.\textsuperscript{50–57} Mitochondria, death receptors, p53, caspases, and endoplasmic reticulum (ER) stress all have been implicated by intervention studies in tubular cell death after ischemia/reperfusion.\textsuperscript{58–63} In this model, Bid connects the death receptor and mitochondrial pathways.\textsuperscript{64}

**NEPHROTOXINS ILLUSTRATE INTRACELLULAR DEATH PATHWAYS**

The study of the molecular mechanisms engaged by nephrotoxins that induce AKI or apoptosis in cultured tubular cells discloses stimulus-specific pathways that lead to specific interventions (Figure 4).\textsuperscript{65} CsA increases Fas expression in tubular cells in culture and in vivo\textsuperscript{66,67}; however, neither neutralizing anti-FasL antibodies nor caspase-8 inhibitors decrease apoptosis induced by CsA.\textsuperscript{67} Similar observations were made with acetaminophen.\textsuperscript{68} This suggests some changes in apoptosis-related molecules are epiphenomena not directly related to cell death. By contrast, Bax-mediated mitochondrial injury and caspase activation are key events in CsA-induced apoptosis of tubular cells.\textsuperscript{67,69,70} CsA induces Bax aggregation and translocation to mitochondria, causing permeabilization of the outer mitochondrial membrane, re-
lease of cytochrome c and SMAC/DIABLO, and activation of caspase-9 and -3. Initiator caspase-2 is also activated and may lead to mitochondrial injury.71–73 In a positive feedback loop, caspases further damage the mitochondria, leading to loss of mitochondrial transmembrane potential (Figure 4A).74 The feedback loop is essential for apoptosis and cell death to proceed because caspase inhibitors prevented both. This is one of several models for the participation of mitochondrial injury in apoptosis.13 Bax antisense oligodeoxynucleotides prevent CsA-induced apoptosis.67 Bax is also required for apoptosis and cell death induced by 3,4-di-deoxyglucosone-3-ene, a toxic glucose metabolite.75 CsA is a potent inhibitor of macrophage apoptosis through the inhibition of inducible nitric oxide synthase, illustrating cell-specific pathways.76 Acetaminophen induces caspase-dependent apoptosis of tubular cells without characteristic mitochon-drial alterations or involvement of Bax.68 Acetaminophen nephrotoxicity is an example of involvement of the ER in apoptosis. ER-initiated apoptosis is triggered by disturbances in calcium homoeostasis or accumulation of misfolded proteins and multiple signaling pathways emerging to promote cell death through caspase-dependent and independent means, including the recruitment of the mitochondrial pathway (Figure 4B).14,77,78 Molecular responses characteristic of involvement of the ER in apoptosis include the expression of CHOP/GADD153, a transcription factor that decreases Bcl-2 levels, and activation of ER-associated caspase-12.79,80 Caspase-12 is present in mice, but most humans carry an inactivating mutation.79,81 Acetaminophen upregulates CHOP/GADD153, leading to caspase-12 cleavage and apoptosis in tubular cells.68 Caspase inhibition by BcXL protects tubular cells from acetaminophen-induced apoptosis but not from eventual cell death.82 In this regard, BcXL interacts with ER proteins such as BAP31, RTN-XS, NSP-C, and the BH3-only protein Spike.83–85 CsA increases CHOP/GADD153 expression but fails to activate caspase-12, suggesting that CHOP up-regulation may be induced by non-ER stressors.86 The ER stressor tunicamycin induces severe histologic tubular injury that is decreased both in CHOP/GADD153 and caspase-12 null mice.79,86 Although these studies serve as a proof of concept for the relevance of ER stress in tubular injury, tunicamycin has no direct clinical relevance. In a more clinically relevant model, ischemia/reperfusion, ORP150 (150-kD oxygen-regulated protein), an inducible ER chaperone, is up-regulated in tubular epithelia and protects in ischemia/reperfusion or hypoxia.81 Aminoglycoside nephrotoxicity is an example of lysosomal participation in apoptosis (Figure 4C).87 Lyosomal accumulation of gentamicin may initially prevent its more toxic cytosolic localization. Eventually, permeabilization of the lysosomal membrane releases free gentamicin to the cytosol and/or releases other lysosomal components that trigger a Bax-mediated mitochondrial pathway of apoptosis.87–89 The proapoptotic role of p53 has been characterized in cisplatin nephrotoxicity (Figure 4D). Cisplatin damages genomic DNA and markedly induces the expression and phosphorylation of p53.90 Pifithrin-α inhibits transcriptional and nontranscriptional activities of p53 and protects tubular cells in culture and in vivo.60,91 p53 transcriptional targets include TRAIL receptors, Noxa, Bax, PUMA, and PIDD.92 The expression of the last two is critical for p53 nephrotoxicity.90,91 PUMA also antagonizes Bcl-xL.91 PIDD promotes the formation of a multiprotein complex, the PIDD-
some, leading to caspase-2 activation, which causes the release of AIF from mitochondria.\textsuperscript{90} Inhibition of p53, caspase-2, or AIF markedly protects from cisplatin-induced apoptosis in cultured tubular cells.\textsuperscript{90} p53 nontranscriptional actions include inactivating Bcl2/BclxL and activating Bax.\textsuperscript{93} In addition, caspase activates mitogen-activated protein kinase.\textsuperscript{94} In the context of cisplatin nephrotoxicity, extracellular signal–regulated kinase promotes apoptosis, contrary to its usual role in cell death regulation.\textsuperscript{94} Cdk2 and E2F1 also participate in cisplatin-induced tubular cell death.\textsuperscript{95,96}

**TARGETING APOPTOSIS**

Survival factors and anticytokine strategies prevent apoptosis \textit{in vivo}.\textsuperscript{21,26,31–35,46} Proof-of-concept studies have also involved genetic manipulation, small interference RNA, or oligodeoxyribonucleotide targeting of different intracellular molecules. In addition, interventions against Bcl2-like proteins and caspases have used cell-permeable peptides. Among antiapoptotic Bcl2 family proteins, Bcl-2 and BclXL have been most extensively studied in the kidney. Bcl2 expression in tubular cells is decreased by several lethal stimuli in cell culture and in AKI.\textsuperscript{28} BclXL is decreased in certain tubular cell populations during AKI and in cultured tubular cells exposed to nephrotoxins such as cisplatin and acetaminophen, serum deprivation, and lethal cytokines such as TNF-α.\textsuperscript{28,82,90} Deprivation of survival factors and TNF-α decreased the expression of mRNA encoding BclXL and protein, whereas acetaminophen increased the proapoptotic degradation of BclXL.\textsuperscript{28,82} This suggests that a decrease in levels of BclXL is a common event in tubular cell death induced by different mechanisms and points to BclXL as a therapeutic target in tubular injury. Indeed, BclXL overexpression protects from acetaminophen and CsA-induced tubular cell death, suggesting that BclXL can defend against apoptosis induced by mitochondrial activation and ER stress.\textsuperscript{82} In addition, BclXL protects from apoptosis induced by lethal cytokines such as TNF-α and FasL.\textsuperscript{28,29} More recently, the cell-permeable BclXL-like molecule TAT-BH4, containing the BH4 domain of BclXL fused to the protein transduction domain of HIV Tat, efficiently prevented apoptosis in cultured cells and \textit{in vivo}.\textsuperscript{97–99} However, it has not yet been studied in the kidney. A KU-70–derived Bax-targeting peptide also protects in tubular cell culture studies.\textsuperscript{75}

Caspases are cysteine proteases that play a central role in apoptosis; however, apoptosis can occur in the absence of caspase activation.\textsuperscript{100} Caspases have nonapoptotic roles in inflammation, cell proliferation, and differentiation that may complicate their therapeutic targeting.\textsuperscript{101} The main role of caspase-1 is to activate inflammatory IL-1β and IL-18, whereas caspase-8 is required for compensatory liver proliferation.\textsuperscript{101,102} Interference with inflammation through IL-18 is instrumental in protection against ischemia-reperfusion injury afforded by caspase-1 deficiency or inhibition.\textsuperscript{103}

Multiple caspases participate in different forms of tubular cell death. Evidence includes demonstration of processing, increased activity, and protective effect of caspase inhibitors; however, prevention of apoptosis by caspase inhibition is not always coupled to increased cell survival. Rather, it may change the form of cell death to necrosis in a stimulus-specific manner. Specific inhibitors of caspase-2, -3, -8, or -9 decrease apoptosis and prolong cell survival in tubular cells exposed to CsA;\textsuperscript{66} however, the same set of inhibitors decrease apoptosis induced by 3,4-di-deoxyglucosone-3-ene but fail to prevent cell death.\textsuperscript{79} In tubular cells exposed to TWEAK, TNF-α, and IFN-γ inhibition of caspase-8 or multiple caspases transforms a weak apoptotic response into massive reactive oxygen species–dependent necrosis.\textsuperscript{45} By contrast, pan-caspase inhibition allows neutrophils exposed to peritoneal dialysis solutions to survive long enough to preserve their antibacterial activity.\textsuperscript{104–106}

\textit{In vivo} caspase inhibitors protect against ischemic injury in brain, heart, and kidney.\textsuperscript{63} The pan caspase inhibitor zVAD prevents the impairment of renal function at an early time point (24 h) when administered at the time of reperfusion.\textsuperscript{62} It is much less effective when administered 2 h later. Longer follow-up studies are needed to exclude the possibility that zVAD is retarding only cell death and favoring more injurious necrosis. In this regard, zVAD exacerbates TNF-α toxicity by enhancing oxidative stress and mitochondrial damage, resulting in hyperacute hemodynamic collapse, kidney failure, and death.\textsuperscript{107} This observation emphasizes the need to understand the intracellular apoptotic pathways that are activated in a cell- and stimulus-specific manner.

**NEW OPPORTUNITIES FOR INTERVENTION**

Small molecules have also been used to inhibit apoptosis. Clinical trials demonstrate the feasibility of the use of caspase inhibition in the clinical setting. Short-term use of IDN-6556, an orally active, liver-specific, pan caspase inhibitor, decreases hepatocyte lysis in patients with chronic hepatitis C.\textsuperscript{108} Its long-term safety remains unclear. IDN-6556 also reduces liver apoptosis in human liver preservation injury and has received an orphan drug label by the US Food and Drug Administration for solid-organ preservation in transplantation.\textsuperscript{109} Other small molecules of interest include the p53 inhibitor pifithrin-α, which prevents apoptosis and protects renal function in ischemia/reperfusion and cisplatin nephrotoxicity, and nanomolecular inhibitors of Apaf-1.\textsuperscript{60,91,110}

The kidney may be a particularly favorable organ for specific targeting of antiapoptotic molecules. OAT1-mediated uptake of cidofovir leads to selective accumulation and toxicity in renal proximal tubular cells.\textsuperscript{111} Similar pharmacokinetics may be used specifically to deliver antiapoptotic drugs to this segment of the nephron. Small molecules may be bound to carriers that lead to specific proximal tubular uptake and organ protection.\textsuperscript{111} Indeed, cidofovir nephrotoxicity is an ideal model for proof-of-concept studies in the regulation of tubular cell apoptosis \textit{in vivo}. The mode of administration of the drug (biweekly intra-
CONCLUSIONS

Accumulating evidence suggests a role for apoptotic pathways in parenchymal cell depletion. There is incomplete understanding, however, of the molecular regulation of apoptosis in renal cells. In particular, stimulus- and cell-specific apoptotic pathways activated during the course of renal disease should be better characterized. Future research should focus on defining the cellular and molecular targets, the optimal time frame, and the specific strategies for therapeutic intervention in various kinds of renal disease. Special consideration should be given to optimizing modes of local delivery of therapies that modulate apoptosis so as to target only specific cell populations during a limited period and limit interference with the process of beneficial apoptosis.114,115

ACKNOWLEDGMENTS

This study was supported by grants FIS 06/0046, SAF03/884, and EU QLG1-CT-2002-01215; Sociedad Española de Nefrología; ISICIII-RETIC REDinREN/RD06/0016; Comunidad de Madrid/FRACM-S-BIO0283/2006; and Programa Intensificación Actividad Investigadora (ISICIII/Agencia Lain-Enralgo/CM) to A.O.

DISCLOSURES

None.

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