Regulated Cell Death in AKI

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ABSTRACT

AKI is pathologically characterized by sublethal and lethal damage of renal tubules. Under these conditions, renal tubular cell death may occur by regulated necrosis (RN) or apoptosis. In the last two decades, tubular apoptosis has been shown in preclinical models and some clinical samples from patients with AKI. Mechanistically, apoptotic cell death in AKI may result from well described extrinsic and intrinsic pathways as well as ER stress. Central converging nodes of these pathways are mitochondria, which become fragmented and sensitized to membrane permeabilization in response to cellular stress, resulting in the release of cell death–inducing factors. Whereas apoptosis is known to be regulated, tubular necrosis was thought to occur by accident until recent work unveiled several RN subroutines, most prominently receptor-interacting protein kinase–dependent necroptosis and RN induced by mitochondrial permeability transition. Additionally, other cell death pathways, like pyroptosis and ferroptosis, may also be of pathophysiologic relevance in AKI. Combination therapy targeting multiple cell-death pathways may, therefore, provide maximal therapeutic benefits.

AKI is a multifactorial and multiphasic renal disease characterized by a rapid decline of renal function, resulting in the accumulation of metabolic waste and toxins and consequent complications and failure of other organs. Clinically, the causes of AKI mainly include sepsis, ischemia-reperfusion (IR) injury, and various endogenous as well as exogenous nephrotoxins. It is estimated that over 2 million people die of AKI each year around the world,[1] and the prevalence of AKI has been increasing rapidly.[1,2] AKI is also known for its association with unacceptably high rates of mortality. For example, in intensive care units, AKI is associated with a mortality rate of 50%–80%. Notably, even if the patients survived, the post-AKI prognosis is dismal, with 7.5% requiring long-term

dialysis and 30%–70% developing complications, including CKD and ESRD. The annual cost of AKI in the United States is estimated to exceed $10 billion.[3,4]

Pathologically, AKI is characterized by renal tubular damage, inflammation, and vascular dysfunction. Injury and death of tubular cells are especially recognized as the precipitating factors in AKI, and as an extension, tubular repair and regeneration are considered major events in kidney recovery from AKI.[5–8] Although sublethal injury is reversible, the death of tubular cells is accompanied by the inevitable loss of the function of the affected cells, and notably it is also frequently the source of damage-associated molecular patterns (DAMPs), the stimulating and amplifying factors of inflammation in tissue damage.[9] In AKI,

various forms of cell death are noticeable: necrosis and apoptosis. This review summarizes the evidence for the various forms of regulated cell death in AKI, delineates their underlying mechanisms with an emphasis on the new insights, and puts forth the perspectives of targeting cell death for the prevention and therapy of kidney injury.

APOPTOSIS—THE CLASSIC VIEW OF REGULATED CELL DEATH IN THE KIDNEY

Evidence for Apoptosis in AKI

In 1992, Schumer *et al.*[10] showed DNA cleavage and nuclear condensation during renal IR injury, showing the first evidence of apoptosis in AKI. To date, the initial observation has been confirmed and extensively expanded. By morphology, apoptotic cells are identified after renal IR by electron microscopy and various nuclear staining methods.[10–12] Biochemically, renal IR leads to the activation

of caspases and endonucleases.[13,14] In addition, regulation of apoptotic genes, including caspases and Bcl-2 family proteins, has been shown.[13–16]

TUNEL staining and the evaluation of *in vivo* cell death. TUNEL has been widely used within the last two decades to evaluate cell death in tissues. Indeed, most dead cells stain positive for TUNEL, because double-strand breaks are found in most programmed cell death. Obviously, TUNEL positivity is found in apoptotic cells, but in contrast to the widespread belief, TUNEL positivity is not limited to apoptosis, because the cells of regulated necrosis are TUNEL-positive as well.[173] Therefore, detection of apoptosis requires additional staining (*e.g.*, cleaved caspase 3 [also referred to as activated caspase 3]) either from tissue lysates in Western blot assays or immhunohistochemically. The difference between TUNEL staining and positivity for cleaved caspase 3 might even be used to quantify the amount of regulated necrosis in tissues,[173] but it cannot further specify the precise pathway of regulated necrosis.[174] To further distinguish necroptosis from apoptosis, one emerging marker for the direct detection of necroptosis is an mAb that detects phosphorylated MLKL.[114] This antibody, however, only detects human phosphorylated MLKL.

Apoptosis is also well recognized in AKI induced by various nephrotoxins. For example, cisplatin is a widely used cancer therapy drug with a major side effect of nephrotoxicity, which limits it therapeutic efficacy.[17,18] Depending on the dosage, cisplatin induces both necrosis and apoptosis in renal tubular cells *in vitro* as well as *in vivo* in animal models. Apoptosis is shown by cell morphology, caspase activation, and terminal deoxynucleotidyl transferase–mediated digoxigenin-deoxyuridine nick-end labeling (TUNEL) assay of DNA damage. Renoprotection against cisplatin nephrotoxicity is associated by the suppression of tubular cell apoptosis,[19–24] further supporting the involvement of apoptosis in cisplatin-induced renal injury. In human kidneys of sepsis-associated AKI, tubular cell apoptosis is detected by TUNEL and activated caspase 3 staining.[25] Of note, in some of the previous studies, apoptosis was detected in kidney tissues by a single method, such as TUNEL assay, which may be questionable for its specificity of apoptosis (Box).

In addition, although tubular apoptosis has often been reported in various models of AKI, the upstream signaling pathways leading to apoptosis in these models can be very different. For example, distinct pathogenic mechanisms may be responsible for apoptosis in ischemic and cisplatin nephrotoxic AKI.[26,27]

In AKI, apoptotic cells are shown in both cortical and medullary regions. In renal tubules, apoptosis occurs in proximal tubules, distal tubules, and tubular cells of the Henle's loop.[10,12,28–36] Numerous renoprotective agents seem to ameliorate AKI, at least in part, by diminishing tubular apoptosis. For example, minocycline, a tetracycline derivative, blocks apoptosis during renal IR, which is accompanied by the amelioration of ischemic renal injury and renal failure.[28,37] Remarkably, deletion of apoptotic genes specifically from proximal tubules results in marked decreases in apoptosis and protection from both ischemic and nephrotoxic AKI.[38,39] Together, these studies show an important role of tubular cell apoptosis in AKI.

Main Pathways of Apoptosis in AKI

Apoptosis can be initiated through several pathways (Figure 1). In the intrinsic pathway, cell stress directly leads to mitochondrial outer membrane permeabilization (MOMP), resulting in the release of apoptogenic factors, including cytochrome c, that then bind Apaf-1 to activate caspase 9. In the extrinsic pathway, ligation of death receptors leads to the recruitment of adapter proteins and subsequent activation of caspase 8.[40] Under endoplasmic reticulum (ER) stress, caspase 12 is activated,[41] and more recent studies suggested that caspase 2 can be an initiator caspase for apoptosis.[42]

All aforementioned apoptotic pathways have been implicated in AKI. The extrinsic pathway of apoptosis mediated by TNF-α and Fas may contribute to tubular cell loss in ischemic and septic AKI.[43–45] Consistently, TNF-α receptor knockout mice are resistant to cisplatin AKI, further supporting the involvement of the TNF-α–mediated extrinsic pathway in the pathogenesis of AKI.[46] In ischemic and cisplatin nephrotoxic AKI, ER stress activation has been documented, but definitive evidence for the involvement in ER stress–related apoptosis has yet to be shown.[47,48] In contrast, the role played by the intrinsic pathway of apoptosis in AKI has been shown convincingly. In 1998, evidence of the activation of the intrinsic pathway in AKI was shown by using the experimental model of hypoxic incubation of renal tubular cells.[49] In this model, cytochrome c is released from mitochondria followed by caspase activation and tubular cell apoptosis. Importantly, the activation of Bax and Bak, two proapoptotic Bcl-2 family proteins, was later confirmed to be key to the mitochondrial leakage or MOMP.[49–51] In animals, MOMP associated by cytochrome c release was shown during ischemia and cisplatin nephrotoxic AKI.[33,37,52] The critical roles of Bax and Bak in AKI have been shown recently using global and proximal tubule–specific gene knockout models.[39] Notably, in humans, mitochondrial damage by Bax and Bak seems to be a key to apoptotic cell death in kidneys injured by ischemia.[53,54] Upregulation of Bcl-2 either pharmacologically or by gene transfection consistently blocks Bax and Bak activation, resulting in the preservation of mitochondrial integrity and cell viability and further supporting a critical role of the intrinsic pathway of apoptosis in tubular injury in AKI.[28,49–51]

Mitochondria and Bcl-2 Proteins: Central Players in Apoptosis

Despite the different initiation mechanisms, most (if not all) apoptotic pathways converge on mitochondria (Figure 1). Although the extrinsic pathway of apoptosis is initiated by ligand binding of death receptors, caspase 8 (after being activated in this pathway) can activate the intrinsic
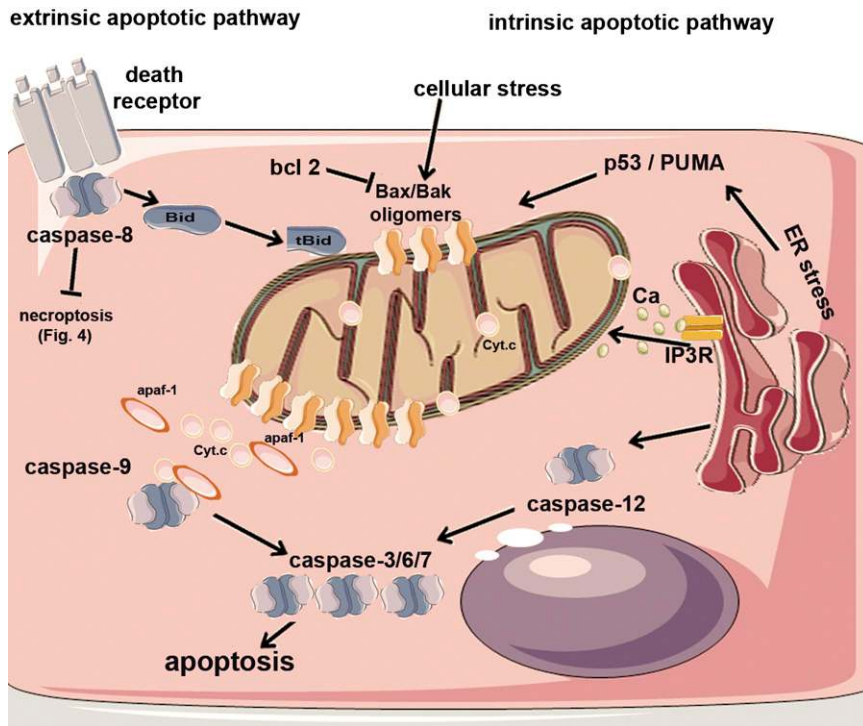
Figure 1. Pathways of apoptosis. In the intrinsic apoptotic pathway, cellular stress leads to the oligomerization of Bax and Bak, an event that permeabilizes the mitochondrial outer membrane, resulting in the release of apoptogenic factors, including cytochrome *c* (Cyt.c). In the cytosol, Cyt.c binds Apaf-1 to recruit and activate caspase 9, which further cleaves and activates executioner caspases, such as caspase 3. In the extrinsic apoptotic pathway, ligation of death receptors leads to the recruitment of adapter proteins and subsequent activation of caspase 8, which further activate executioner caspases and prevent necroptosis (Figure 4). Active caspase 8 also cleaves Bid to its truncated form tBid, which translocates to mitochondria to activate the intrinsic pathway to amplify the apoptotic cascade. In the ER stress pathway, caspase 12 mediates the activation of executioner caspases. ER stress may activate the intrinsic apoptotic pathway through Ca^{2+} signaling and the induction of proapoptotic Bcl-2 family proteins, such as PUMA. IP$_3$R, inositol trisphosphate receptor.

pathway of apoptosis through Bcl-2 family proteins.[55,56] Mitochondria also play an important role in apoptosis initiated by ER stress and caspase 2.[57,58] Thus, mitochondrial damage characterized by MOMP is considered a central control point of apoptosis.[59,60]

At the molecular level, Bcl-2 family proteins are the key regulators of mitochondrial integrity. Defined by the presence of Bcl-2 homology (BH) domains, Bcl-2 family proteins can be proapoptotic or antiapoptotic.[59,61–63] Specific functions of individual Bcl-2 proteins are dictated by the organization of the BH domains. Accordingly, antiapoptotic members, like Bcl-2 and Bcl-X$_L$, usually contain four BH domains. Proapoptotic members can be further divided into two groups: multi-BH domain proteins, such as Bax and Bak, and BH3-only proteins, such as Bid and PUMA.[59,61–63] Antiapoptotic Bcl-2 proteins protect cells by preserving the integrity of mitochondria, whereas the proapoptotic molecules kill cells by permeabilizing the organelles.[59,61–63] Notably, Bax and Bak provide a requisite gateway to mitochondrial injury in various apoptotic models.[64] Deletion of Bax and/or Bak consitently leads to a marked resistance to tubular apoptosis in AKI.[39,65]

Despite the significance of Bax and Bak in mitochondrial injury during apoptosis, the mechanism underlying their activation remains elusive. Normally, Bax is cytosolic, whereas Bak resides on the mitochondrial outer membrane. On cell stress or apoptosis, Bax translocates to mitochondria, inserts to the outer membrane, and forms oligomers. Meanwhile, Bak is also activated to oligomerize. Bax activation may involve the interaction with specific proteins, such as Bid, p53, humanin, 14-3-3 protein, ku70, and Bif-1.[66–72] In renal tubular cells, Borkan and colleagues[73] have recently shown the interaction of nucleophosmin with Bax, which seems to be critical to Bax activation during metabolic stress *in vitro* and ischemic AKI *in vivo*. As discussed below, the activation of Bax and Bak also involves a striking change of mitochondrial morphology and consequent alterations of the membrane property.

Mitochondrial Dynamics in Apoptosis and AKI

Mitochondrion, of the Greek mito for thread and chondro for grain, assumes distinct morphologies. Recent research has further revealed that mitochondria are a class of highly dynamic organelles.[74,75] Whether mitochondria display a filamentous morphology or punctiform is determined by two opposing processes: fission and fusion. Accordingly, fusion of mitochondria promotes a filamentous network, whereas fission fragments mitochondria into short rods or spheres. In mammalian cells, mitochondrial fusion involves mitofusin-1, mitofusin-2, and OPA1, whereas fission depends on Drp-1, Fis-1, and others.

In 2001, Youle and colleagues[76] reported that mitochondrial dynamics are lost on cell stress or apoptosis, leading to the fragmentation of mitochondria. Importantly, inhibition of mitochondrial fragmentation blocks cytochrome c release and apoptosis. *In vivo* in diseases, the role of mitochondrial fragmentation was first suggested by using experimental models of ischemic and cisplatin nephrotoxic AKI. Under these conditions, Drp1 translocates to mitochondria, which is followed by sequential events of mitochondrial fragmentation, Bax/Bak activation, cytochrome c release, caspases activation, and apoptosis. Blockade of Drp1 pharmacologically or genetically preserves the filamentous morphology of mitochondria, resulting in the suppression of tubular cell apoptosis and amelioration of AKI.[77]

These findings have been extended to other disease conditions, such as IR injury in the heart and brain,[78,79] supporting mitochondrial fragmentation as a general pathogenic mechanism.

Mitochondrial fragmentation is the result of the disruption of mitochondrial dynamics. On cell stress, Drp-1 is activated (as indicated by its translocation to mitochondria) to accelerate fission. The regulation of Drp1 involves multiple post-translational modifications. In models of AKI, Drp1 is rapidly dephosphorylated in renal tubular cells, likely through calcineurin family phosphotases.[80] Prevention of Drp1 dephosphorylation by calcineurin inhibitors can partially block mitochondrial fragmentation, cytochrome c release, and apoptosis, supporting a role of the dephosphorylation in Drp1 activation and mitochondrial fission.[80] Interestingly, mitochondrial fragmentation not only involves hyperactivation of fission but also, depends on the arrest of fusion. Intriguingly, fusion arrest is governed by the interaction of mitofusins with Bak.[81] Normally, Bak binds both mitofusin-1 and mitofusin-2. On cell stress or apoptosis, Bak dissociates from mitofusin-2 and binds mitofusin-1 at a higher affinity. Remarkably, a Bak mutant incapable of dissociating from mitofusin-2 cannot induce mitochondrial fragmentation,[81] suggesting that Bak contributes to mitochondrial fragmentation by switching its binding from mitofusin-2 to mitofusin-1 (Figure 2). This key finding has been confirmed in experimental models of oxidative lung injury.[82] A role of Bak in mitochondrial fragmentation has further been shown *in vivo* in AKI models by using gene knockout mice.[39] Thus, mitochondrial fragmentation is a combined result of fission activation by Drp1 and fusion arrest mediated by Bak interaction with mitofusins.

Of note, mitochondrial fragmentation is initially reversible; in other words, fragmented mitochondria can refuse if the insult is removed from the cell in time. Then how can mitochondrial fragmentation, a seemingly morphologic change, affect mitochondrial injury? The answer may be in the changes of mitochondrial membrane properties that occur as a result of the

loss of mitochondrial dynamics. It has been shown that fragmented mitochondria are sensitized to Bax insertion and oligomerization.[83] Thus, mitochondrial fragmentation may participate in apoptosis by facilitating Bax insertion and oligomerization, resulting in outer membrane permeabilization and leakage of apoptogenic factors, such as cytochrome c. In addition, mitochondrial fragmentation may contribute to mitochondrial permeability transition (MPT) at the inner membrane, resulting in necrosis (Figure 2). In kidneys, mitochondrial fragmentation as a result of the disruption of mitochondrial dynamics contributes to not only AKI but also other renal diseases, including diabetic nephropathy.[84,85]

REGULATED NECROSIS

Necrosis is distinguished from apoptosis by the breakdown of the integrity of the plasma membrane. As such, necrotic cell death is accompanied by the release of unprocessed intracellular content, including cellular organelles, highly immunogenic proteins, like IL-33, F-actin, ATP, IL-1α, and HMGB1, double-stranded DNA, and RNA.[86] These proimmunogenic cellular components are collectively
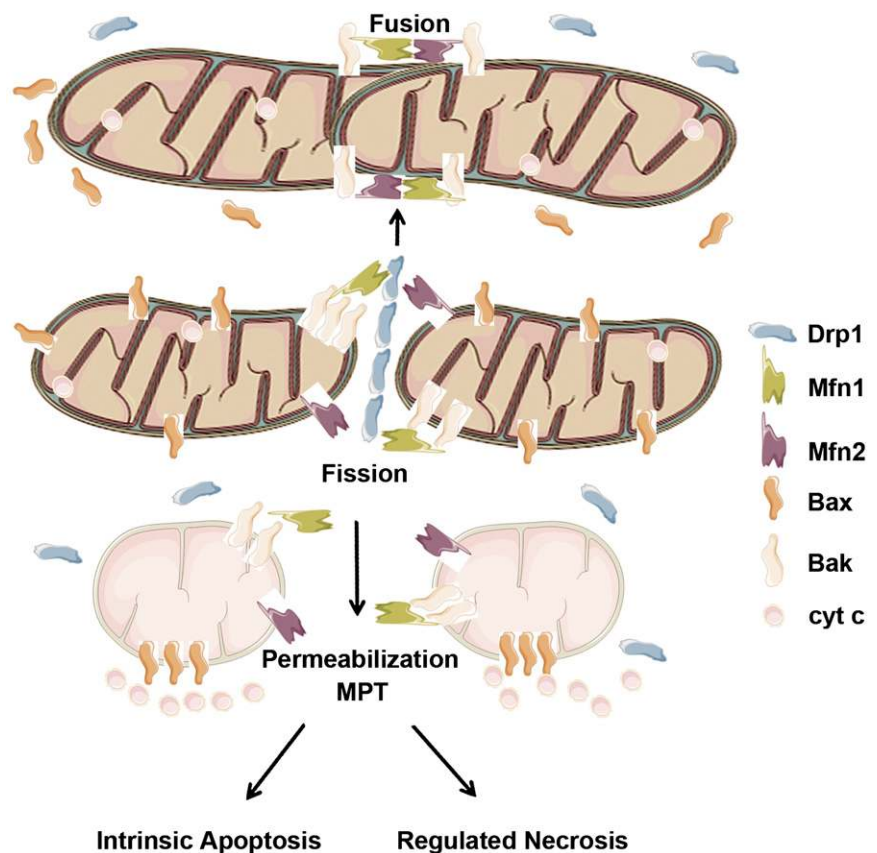

Figure 2. Mitochondrial dynamics in apoptosis. Under normal *in vivo* conditions, Bax and Drp1 are located within the cytosol, whereas Bak is at the mitochondrial outer membrane, where it binds both mitofusin-1 (Mfn1) and mitofusin-2 (Mfn2) to maintain mitochondrial fusion, ensuring its filamentous morphology. On cellular stress, Drp1 translocates to mitochondria, where it forms a restriction ring to activate the cleavage of the organelles; meanwhile, Bak dissociates from Mfn2 to bind Mfn1, which leads to an arrest of fusion and mitochondrial fragmentation. Fragmented mitochondria are more sensitive to Bax oligomerization, resulting in outer membrane permeabilization followed by the release of apoptogenic factors, such as cytochrome c (cyt.c), to activate the intrinsic apoptotic pathway. In addition, mitochondrial fragmentation may also contribute to MPT, leading to necrosis.

referred to as DAMPs.[86,87] The dynamics of DAMP release in the kidney have recently been shown by intravital microscopy.[88] Although originally thought to be accidental, recent work has revealed several pathways of genetically determined and regulated necrosis[89,90] (Figure 3), and we are beginning to understand the relative contribution of these pathways with presumably overlapping function (see below). On the molecular level, the best characterized pathway of regulated necrosis is necroptosis, an receptor-interacting protein kinase–based necrotic cell death.[91]

Necroptosis—a Paradigm Shift

The *in vivo* relevance of necroptosis has been undoubtedly clarified by the rescue of caspase 8–deficient mice by ablation of receptor-interacting protein kinase 3 (RIPK3).[92,93] Whereas caspase 8–deficient mice die at day 10.5 *in utero* and RIPK3-deficient mice have been described without phenotype,[94] caspase 8/RIPK3 double-deficient mice are born at expected Mendelian frequencies and show a phenotype that was previously described as lymphoproliferation or generalized lymphoproliferative disease in mice that carry mutations in the Fas-Fas ligand pathway.[92,93] From these groundbreaking experiments, it became obvious that the most important function of caspase 8 is the prevention of necroptotic cell death by a caspase 8/FLIP long heterodimer[93,95] rather than the induction of either apoptosis by the caspase 8 homodimer[96,97] or inflammation, possibly mediated by single caspase 8 molecules.[98] In addition, from these studies, an *in vivo* model emerged that allows for direct investigation of mice deficient in extrinsic (receptor-mediated) apoptosis.

The signaling pathway of necroptosis has been reviewed in detail recently.[90,99] Briefly, the main events that trigger necroptosis are engagement of death receptors in the presence of caspase inhibition,[100,101] stimulation of Toll-like receptors,[92,102] signaling through interferons,[103,104] or recognition of intracellular viruses by the protein DAI.[105,106] Any of these initial triggers uses an receptor-interacting protein–homotypic interacting motif (RHIM) domain to activate the kinase RIPK3,
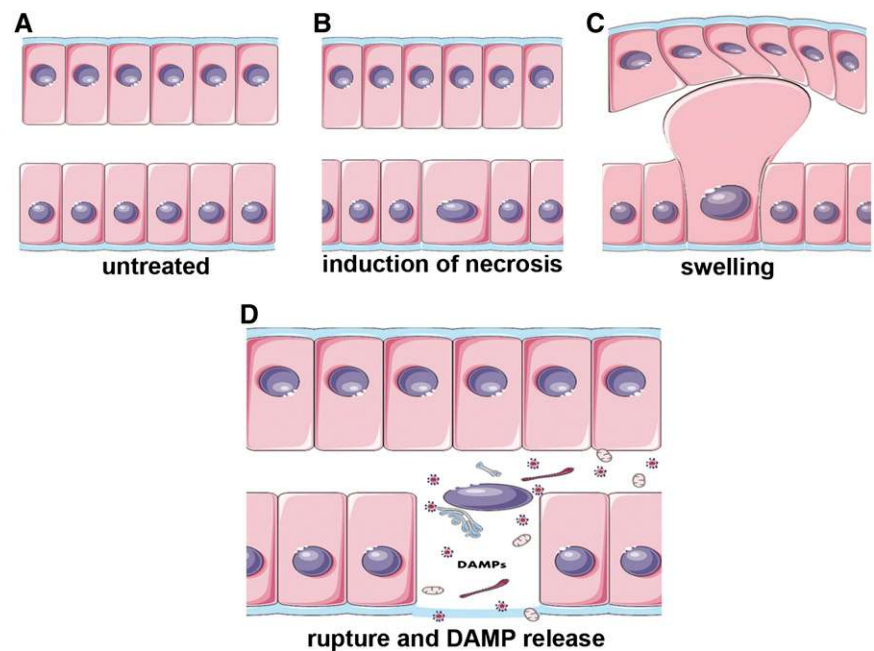

Figure 3. Necrotic cell death in renal tubular epithelial cells. Four-phase model of necrosis-associated release of DAMPs. (A) Tubular epithelial cell layer under physiologic conditions. (B) On induction of regulated necrosis as the common mechanism of several distinct intracellular signaling pathways, individual cells begin to swell on specific genetically-determined intracellular programs that decode for regulated necrosis (like necroptosis, MPT-mediated regulated necrosis, pyroptosis, or ferroptosis). (C) Severe swelling of the luminal part of a tubular cell that undergoes regulated necrosis. (D) Plasma membrane rupture associated with DAMP release. Regulated necrosis might, therefore, trigger subsequent detrimental immune responses that cause additional organ damage beyond the primary loss of function from cell death. In transplanted organs, a classic setting for IR injury, DAMPs released from necrotic cells might trigger rejection, despite the state of immunosuppression.

an essential mediator of necroptosis,[107–109] which itself contains an RHIM domain and phosphorylates the downstream pseudokinase[110] mixed lineage kinase domain–like (MLKL).[111,112] Phosphorylation of MLKL by RIPK3 leads to a molecular switch mechanism, which exposes the N-terminal portion of MLKL[113] to induce plasma membrane rupture.[114]

In the kidney, necroptosis was first suggested in renal ischemic AKI by showing a protective effect of necrostatin-1 (Nec-1), an inhibitor of RIPK1 (the *bona fide* upstream activator of RIPK3 in the necroptosis pathway) (Figure 4).[115] Other groups have subsequently confirmed a role for Nec-1 in tubular cells,[116–118] and the protection of RIPK3-deficient mice was shown in ischemic and cisplatin-induced AKI.[27] Importantly, caspase 8/RIPK3–double knockout mice did not provide additional protection in the ischemic model but did in

the cisplatin model, suggesting that, unlike previously suggested,[119] extrinsic apoptosis may be of minor importance in IR injury but significantly contributes to cisplatin-induced AKI.[27] This assumption is further underscored by the absence of a protective effect of the pancaspase inhibitor zVAD in the model of IR injury,[115] whereas zVAD does prolong overall survival after a lethal bolus of cisplatin.[27] In support of this concept, cyclosporin-mediated tubular damage[120] or contrast-mediated AKI[121] have been reported to be prevented by Nec-1. It should be mentioned, however, that, in these two cases, a clear detection of cell death failed and that it cannot formally be excluded that Nec-1, other than its obvious effects on necroptotic cell death, might also affect peritubular blood flow by uncharacterized means. In this sense, it is noteworthy that the highest expression of RIPK3, which is thought to indicate the
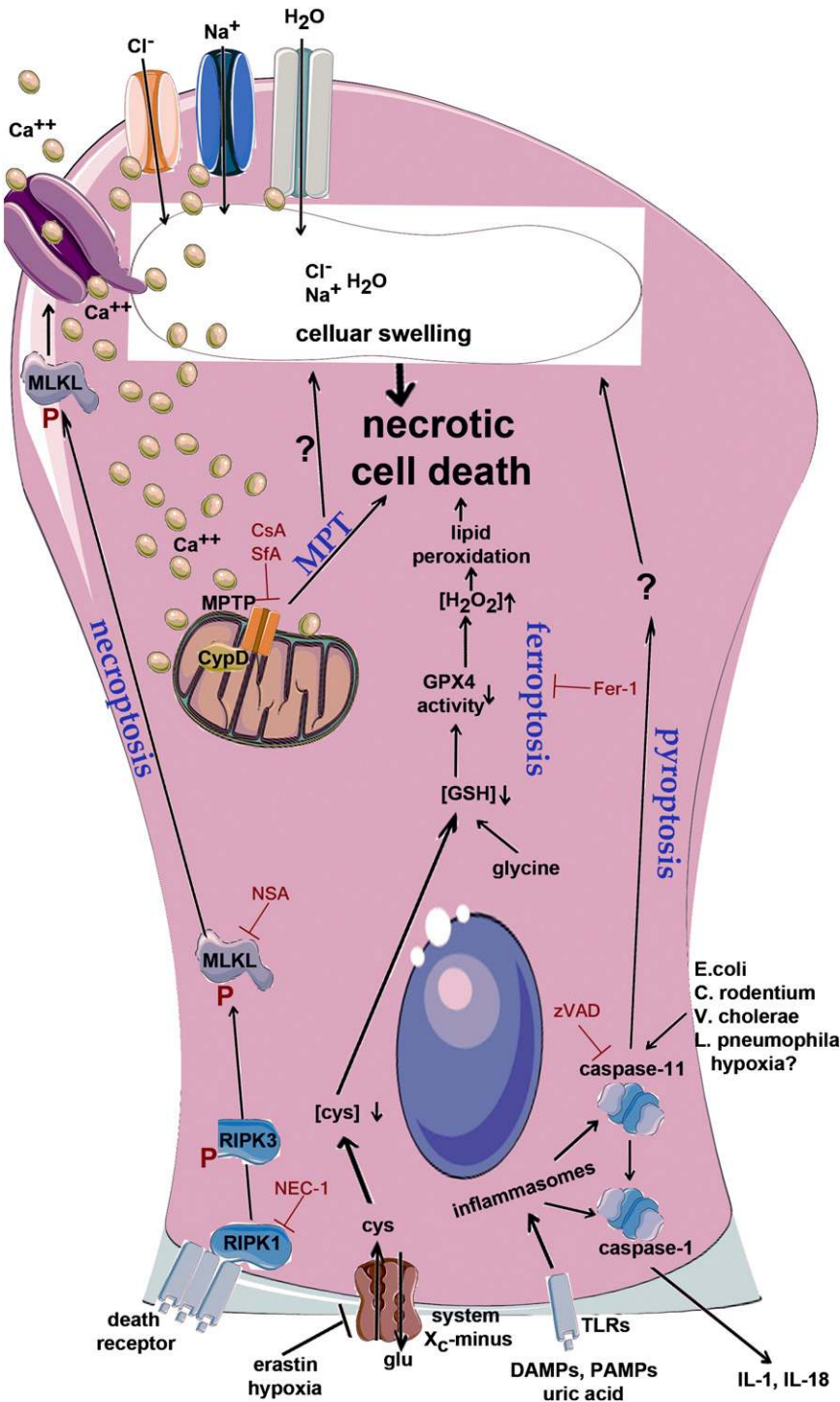
Figure 4. Model of the integrated molecular signaling pathways of regulated necrosis in renal tubular cells. Four separate pathways of regulated necrosis may contribute to the overall organ damage in AKI. The common downstream mechanism that precedes necrotic cell death is apical swelling, which ultimately induces plasma membrane rupture as recently shown by intravital microscopy. RIPK1/RIPK3-dependent necroptosis has been extensively investigated in the kidney and is triggered by death receptors. RIPK3 is activated by phosphorylation and in turn, phosphorylates the pseudokinase MLKL, which has been suggested to be involved in the opening of plasma membrane calcium channels. Calcium-activated chloride and sodium channels may subsequently open to increase NaCl permeability,

cellular sensitivity of necroptosis,[108] was found in glomerular endothelial cells rather than tubular cells.[115]

Recently, RIPK3-deficient mice have been investigated in the model of adriamycin (ADR)-induced podocytes injury.[122] As expected from previous investigations on the level of RIPK3 expression in podocyte cell lines,[115] RIPK3 deficiency did not prevent ADR-induced proteinuria compared with wild-type mice but strongly prolonged overall survival in this model, unless the highest concentrations of ADR were used. Obviously, ADR-induced lethality depends on necroptosis, but it must be concluded that survival in this model is not dependent on podocyte injury, questioning the usefulness of this survival readout for podocyte damage. Taken together, necroptosis has, therefore, been shown to be critically involved in nephrotoxicity of cisplatin, cyclosporin, and ADR as well as, most notably, renal IR injury and kidney transplantation.

Necrosis by Mitochondiral Permeability Transition

Mitochondria are of outstanding interest and significance in cell death research.[123,124] As outlined above, MOMP is regarded as the point of no return in the life and death decision during apoptosis.[123,125] Interestingly, mitochondrial dysregulation, particularly in the form of MPT, is also capable of inducing necrotic cell death[126] (Figure 2). MPT is a process that leads to the sudden exchange of solutes between the cytosol and the mitochondrial matrix through an elusive MPT pore (MPTP) that spans both the inner and outer mitochondrial membranes. The molecular composition of the MPTP remains elusive and a matter of debate.[126–128] It is, however, generally accepted that the opening of the MPTP is regulated by the matrix protein cyclophilin D (CypD). In line with this finding, CypD-deficient mice have been shown to be protected from ischemic AKI.[27,129,130] In addition, these mice are protected from cisplatin-induced AKI.[27] Precise mechanisms about the regulation of the CypD-mediated opening of the MPTP remain unclear. In this regard, p53 has recently been suggested to be involved,[131,132] but such reports are under debate.[133] MPT

was long known to be potentially targeted by either cyclosporin or sanglifehrin A *in vitro* and *in vivo*,[27,134–136] and such effects might be of outstanding clinical importance, because trials have revealed a cyclosporin-sensitive role for MPT in myocardial infarction[137]; however, unfortunately, larger follow-up studies are still lacking. In addition, it is worth mentioning that there is yet another pathway of regulated necrosis called parthanatos[89] because of its dependency on the nuclear protein PARP1.[90] The *in vivo* relevance of parthanatos has been made very clear in both unilateral urethral obstruction and ischemic AKI.[138,139] Future work on the potential overlay of parthanatos with MPT will be extraordinary helpful for untangling the complex web of interconnected pathways of regulated necrosis.

Pyroptosis—Maximal Immunogenicity of Necrosis

Pyroptosis is a necrotic-type cell death that was thought to occur exclusively in macrophages,[140,141] but recent reports find comparable features in T lymphocytes,[142] neurons,[143] and tubular epithelial cells.[144–147] The unique feature of pyroptosis compared with other pathways of regulated necrosis is the maturation of proinflammatory cytokines during the cell death process,[148–150] which depends on cleavage mediated by nonapoptotic caspases. Many of the *in vivo* studies on pyroptosis have been performed in caspase 1–deficient mice, which have been described to carry a passenger mutation that functionally renders them caspase

1/11 double-deficient.[151] It was not until the report of caspase 11–deficient mice that it was realized that caspase 11 mediates the pyroptotic cell death, whereas caspase 1 is thought to be mainly responsible for pro–IL-1β and pro–IL-18 cleavage.[90] How caspase 11 mediates the downstream molecular events required for pyroptosis remains unclear, but in some similarity to necroptosis, it is speculated that plasma membrane channels are involved in the terminal cellular swelling.[140] Caspase 11–deficient mice have not yet been studied in kidney diseases, including AKI; however, there are *in vitro* data pointing to this direction.[145] Pyroptosis may be targeted by caspase inhibitors or cytokine response modifier A in the case of caspase 1.[152]

Ferroptosis—Iron-Dependent Necrosis

While searching for novel ways to kill tumor cells, Stockwell and colleagues[153] identified a compound named erastin that induces necrotic cell death in highly resistant RAS-transformed cancer cells. Following this path, the previously unrecognized pathway of regulated necrosis turned out to be dependent on iron and was found to involve glutathione metabolism. A plasma membrane Cys/Glu exchanger (termed system Xc-minus) was identified to fuel cells with cysteine, which enables glutathione synthesis required for the reactive oxygen species–eliminating action of glutathione peroxidase 4.[154] This enzyme removes H_2O_2 to prevent intracellular lipid peroxidation, which might directly affect,

among others, lysosomal membranes and lead to lysosomal membrane permeabilization. With the detection of these molecular events, ferroptosis turned out to be a druggable pathway of regulated necrosis through the interference with the small molecule ferrostatin-1.[155] With respect to kidney tubular cells, first results from kidney tubular cell lines treated with *tert*-butylhydroperoxide[116] and freshly isolated tubules challenged with iron and hydroxyquinoline in the presence of ferrostatin-1 strongly increased cellular survival.[156] Additional light has been shed on the role of iron in the pathophysiology of AKI from experiments using proximal tubular cell–specific ferritin heavy chain–deficient mice, which were shown to be sensitive to cisplatin-induced AKI and rhabdomyolysis-induced AKI.[157] In addition, renal cell carcinomas were by far the most sensitive in a panel of 60 cancer cell lines from eight tissues tested with erastin.[154] Therefore, ferroptosis is one of the promising therapeutic targets, especially in diseases dominated by kidney tubular necrosis,[90,158] like ischemic, cisplatin nephrotoxic, and rhabdomyolysis-induced AKI.[159] In some of these models, iron chelators have been investigated long before the detection of ferroptosis, but those compounds, such as desferoxamine, never made it into the clinical routine, despite considerable effects in *ex vivo* experiments with kidney tubules. It will, therefore, be of importance to re-evaluate renal data generated with desferoxamine in light of the molecular understanding of ferroptosis.

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which may then cause water influx, cellular volume expansion, and plasma membrane rupture. Ca^{2+} from this source and others might also be involved in MPT, which directly causes regulated necrosis by unknown means. MPT and necroptosis have been clearly shown to exhibit two separate pathways in AKI. Inhibition of the cell surface cystine/glutamate antiporter system Xc-minus depletes intracellular cystine, which is reduced to cysteine on the intracellular side and together with glycine and glutamate, required for the synthesis of glutathione (GSH). Permanent hydrogen peroxide (H_2O_2) synthesis, especially in stressed cells, requires the GSH-dependent activity of glutathione peroxidase 4 (GPX4) to prevent H_2O_2-mediated lipid peroxidation followed by necrotic cell death. Finally, Toll-like receptors (TLRs) are activated by DAMPs or pathogen-associated molecular patterns (PAMPs) and crystals. TLRs activate inflammasomes, which induce caspase 1–dependent maturation of the proinflammatory cytokines IL-1β and IL-18. In parallel, inflammasomes trigger caspase 11–mediated cellular swelling and necrotic cell death, which is referred to as pyroptosis. The combination of DAMP release by necrosis and cytokine maturation renders pyroptosis an even higher immunogenic entity compared with the other pathways of regulated necrosis. Necroptosis can be prevented by the RIPK1 inhibitor Nec-1, its more stable variant, or the human-specific MLKL inhibitor necrosulfonamide (NSA). MPT can be inhibited by cyclosporin (CsA) or sanglifehrin A (SfA), ferroptosis is blocked by the small molecule ferrostatin-1 (Fer-1), and broad spectrum caspase inhibitors (*e.g.*, zVAD-fmk) interfere with pyroptosis signaling. C. rodentium, *Citrobacter rodentium*; cys, Cysteine; *E. coli*, *Escherichia coli*; glu, glutamate; *L pneumophila*, *Legionella pneumophila*; *V. cholerae*, *Vibrio cholerae*.

RELATIONSHIP BETWEEN APOPTOSIS AND REGULATED NECROSIS

As discussed above, apoptosis and regulated necrosis are characterized by distinguished morphologic, cell biologic, and biochemical features. However, these two forms of regulated cell death are not mutually exclusive, and in many pathologic conditions, including AKI, apoptosis and necrosis coexist. It remains unclear as to what determines if a given cell will die by apoptosis or necrosis.

Apoptosis has been discussed to cause secondary necrosis. However, apoptotic cells may break down their plasma membrane during prolonged injury, displaying a necrotic morphology *in vitro*. *In vivo*, apoptotic cells and their debris are thought to be rapidly removed by phagocytic cells.[160–163] However, traditional apoptotic signaling, when intercepted, may be diverted to necrosis. This result is well exemplified by necroptosis, in which death receptors are activated but caspase 8 is blocked by pharmacologic or viral inhibition,[89,90] resulting in RIPK3-mediated phosphorylation of MLKL and necroptosis.

Apoptosis and regulated necrosis may also interact at various molecular and cellular levels. Clearly, as presented above, both forms of cell death involve pathologic changes in mitochondria. Moreover, Bax is known as a classic proapoptotic Bcl-2 protein that permeabilizes mitochondrial membrane to release apoptotic factors for intrinsic apoptosis. However, Bax has recently been implicated in the regulation of MPT-related necrosis by affecting mitochondrial dynamics.[164] In AKI, the involvement of Bax in necrosis may depend on experimental models. Although Bax-null mice showed less tubular apoptosis and necrosis in cisplatin nephrotoxic AKI,[65] Bax ablation only has significant effects on tubular apoptosis in ischemia AKI.[39] Recent work also showed that RIPK3, the key player in necroptosis, is capable of promoting apoptosis when the kinase function of RIPK3 is lost *in vivo*[165] or in complex *in vitro* settings, in which cellular inhibitors of apoptosis or the kinase TAK1 are inhibited.[166,167] Following this thought, the effectiveness of

RIPK3 kinase inhibitors seems to depend on the interaction between RIPK1 and RIPK3 through their RHIM domains.[102] Although these interactions between apoptosis and necroptosis have been worked out in detail, very limited data are available on the interaction between apoptosis and other pathways of regulated necrosis, especially pyroptosis, which is hard to investigate because of the limited specificity of caspase inhibitors[168] and the dependency of both of these pathways on caspases.[90] It is important to understand the complex interplay of the pathways of regulated cell death, especially with the therapeutic idea to target these pathways.

In AKI, very limited information is available on functional or morphologic consequences of interference with apoptosis on other pathways of regulated cell death or *vice versa*.[115] Moreover, the relationship between various forms of cell death in AKI remains to be examined. However, regardless the etiology, AKI is known to involve mixed forms of regulated cell death, and, as presented above, suppression of one form of cell death may have significant renoprotective effects, which nonetheless, are mostly incomplete. The contributions of different forms of cell death in AKI also depend on the nature (ischemic, nephrotoxic, or septic AKI) and severity of the injury. It is important to understand the relative contributions and the potential redundancy of the cell-death pathways to guide the therapeutic strategies for AKI therapy as well as consider the obvious immunologic consequences on the basis of the release of DAMPs from necrotic cell death.[169]

TARGETING RENAL CELL DEATH FOR AKI THERAPY

To date, >1400 PubMed-listed studies on "apoptosis and kidney" or "apoptosis and renal" have been published; unfortunately, no apoptosis-targeting approach has been made into clinical routine in any field (not restricted to the prevention of AKI). Unquestionably, apoptosis is involved in pathologic conditions in kidneys, notably AKI, but whether apoptosis

significantly contributes to functional organ failure was recently questioned, because caspase inhibitors (zVAD-fmk, q-VD, and zIETD-fmk) are not efficacious in blocking AKI.[27,115] In addition to inhibiting caspases, these inhibitors may affect other cell-death/survival-regulatory pathways, such as autophagy.[170] Moreover, at least for death receptor–independent intrinsic apoptosis, inhibition of caspases is at a downstream level of apoptosis, and without blocking upstream apoptotic events, such as those at mitochondria, the viability of renal tubular cells is ultimately lost. In this regard, intrinsic apoptosis is not completely prevented in the presence of caspase inhibitors, like q-VD,[171] which raises important questions as to how to target apoptosis for the prevention and treatment of AKI. It is noteworthy that, in AKI, apoptosis does not occur immediately or at one time point; rather, it persists in kidney tissues for days to weeks after injury. For example, in ischemic AKI in mice, tubular apoptosis starts a few hours after reperfusion, reaches the maximal level at 24–48 hours, and lasts for days. Thus, apoptosis, like regulated necrosis, is a continuous process in the disease condition. Accordingly, apoptosis is detected in a relatively low percentage of tubular cells in a snapshot fashion or at any given time points. Nonetheless, the cumulative number of apoptotic cells may become remarkable. In most clinical settings of AKI, patients have passed the initial injury phase; if tubular apoptosis was still occurring at the time of diagnosis, there would be a chance for apoptosis-targeting therapy to prevent additional deterioration of tissue and renal function and time for kidney repair. Additional *in vivo* investigations with techniques, such as intravital microscopy, may gain insights into the dynamics of apoptosis in AKI, and, more importantly, upstream apoptotic events should be targeted for effective therapy.

Obviously, as outlined in detail above, apoptosis is clearly involved in several pathologic conditions in the kidney, but significant prevention of the primary pathology seen in most preclinical conditions of AKI, necrosis, and functional markers of AKI has not been reproducibly reported through the addition of

inhibitors of apoptosis. This finding is in line with the low levels of detection of cleaved caspase 3 in lysates taken from injured kidneys and rarely cleaved caspase 3–positive cells in immunohistochemistry. When it was realized in 2005 that regulated necrosis might serve as a therapeutic target by the identification of Nec-1,[172] high hopes were raised for necroptosis-targeting strategies. It was, therefore, disappointing to realize that Nec-1 could only partially protect from ischemic AKI[115] and that other pathways might be of importance other than necroptosis. Consequently, because combination therapy seems to be more effective,[27] the protection is still incomplete, leaving significant histologic damage and DAMP release. Additional combinations may be useful (*e.g.*, the addition of ferroptosis inhibitor), but it must be kept in mind that the translation of such results into clinical trials is highly problematic; control groups are required for any single- and double-therapeutic strategy, and support of such studies might become long-winded in the absence of strong, convincing preclinical evidence. In addition, for necroptosis, it is understood that plasma membrane rupture occurs as early as 20 minutes after RIPK3 dimerization,[91] and application of Nec-1 30 minutes after the beginning of reperfusion has no detectable protective effect.[115] Therefore, targeting regulated necrosis may be limited to such disorders in which AKI may be anticipated, like heart surgery–associated AKI, contrast-induced AKI, or kidney transplantation.

In conclusion, tubular apoptosis has been shown unequivocally in various types of AKI, including in diseased human kidneys, but despite 20 years of intensive research, an apoptosis-targeting strategy has not found its way into clinical routine. Recent work has further implicated different forms of regulated necrosis in AKI. Although significant advances have been made in the understanding of the cellular and molecular basis of cell death, targeting of signaling pathways of regulated necrosis for therapy has not yet been investigated in other than promising preclinical settings. Strategically, it is clear now that specific therapeutics have to block upstream events

of cell death. In this regard, mitochondria, the converging point of cellular injury and death, may be a promising target of therapy, but mitochondria are not involved in some pathways of regulated necrosis, such as necroptosis, which are clearly relevant in AKI. Therefore, in view of the many subroutines of cell death in AKI, it is necessary to consider combination therapies that block multiple pathways of regulated cell death simultaneously or at different time points to ensure cell survival and renal function.

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