CHAPTER 20

Cell Death Pathways: Apoptosis and Regulated Necrosis

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OBJECTIVES

This chapter will:

1. Present a summary of the various known mechanisms of apoptosis and regulated necrosis.
2. Discuss the mechanism underlying regulated cell death in acute kidney injury (AKI).
3. List the evidence for regulated cell death in human AKI.
4. Consider potential therapeutic strategies to manipulate regulated cell death in human AKI.

The definition of acute kidney injury (AKI) has evolved over the past decade in an attempt to establish a standardized classification system for the purposes of clinical practice and research. This has been inherently challenging because AKI is a heterogenous condition that can result from multiple causes, and because with the exception of very recent advances that are not yet fully ingrained in clinical practice, available biomarkers such as urine output and creatinine are limited by lack of specificity to underlying mechanisms and by phase lag between onset of injury and clinical detection, respectively. However, current available classification systems such as the risk, injury, failure, loss, and end-stage renal disease (RIFLE), AKI Network (AKIN), and Kidney Disease: Improving Global Outcomes (KDIGO) classifications continue to stage severity of AKI based on increased plasma creatinine and/or diminished urine output.[1] One of the major limitations in the study and understanding of the mechanisms leading to AKI has been the lack of pathology specimens, because in the clinical setting, performing biopsies for diagnostic purposes poses an unjustifiable risk to the patient. However, studies using postmortem biopsies of renal tissue have started to provide some histopathologic evidence of what AKI in different clinical circumstances (but mainly sepsis) looks like. Because of the varying mechanisms of injury with potential different injury sites that can lead to AKI, advancements in our knowledge of cell death in AKI are necessary to truly understand the processes leading to kidney injury and develop therapeutic management strategies.

MECHANISMS OF CELL DEATH

The Nomenclature Committee on Cell Death (NCCD) broadly classifies cell death into two distinct categories, accidental cell death (ACD) and regulated cell death (RCD), and last published their recommendations on classifications of cell death types in 2015.[2] Severe mechanical, physical, or chemical factors such as trauma and extremes in temperature or pH can lead to ACD. Because this type of cell death results from harsh conditions, it is thought to be not amenable to modulation. However, dying cells release endogenous damage-associated molecular patterns (DAMPs), which can cause an innate inflammatory response and can lead to further injury and death of cells that had survived the original insult.[3] Regulated cell death, on the other hand, refers to multiple genetically encoded pathways, summarized later, that are initiated in an attempt to maintain cell homeostasis; it includes a subtype called programmed cell death (PCD), which describes RCD that occurs as part of development or to preserve physiologic tissue homeostasis.[2]

REGULATED CELL DEATH

Early classifications of cell death were based on morphologic changes and included the distinct classification of cell death as apoptosis, autophagy, or necrosis.[4] Apoptosis was viewed as synonymous with PCD and necrosis with accidental cell death. However, morphologic features cannot necessarily be used to determine the functional aspects of cell death, and modern classifications now also include molecular and biochemical measures.[5] A summary of the morphologic and biochemical features of apoptosis versus necrosis is shown in Table 20.1.

Apoptosis

Apoptosis is a term used to describe an adenosine triphosphate (ATP)-dependent form of cell death characterized by shrinkage of the cytoplasm, chromatin condensation, nuclear DNA fragmentation, plasma membrane blebbing, and the formation of apoptotic bodies followed by elimination of the dead cells by phagocytes. It generally is not associated with inflammation. Biochemical criteria include phosphatidylserine (PS) exposure, a phagocytic signal on the cell surface, changes in mitochondrial membrane permeability, release of mitochondrial intermembrane space proteins, and caspase-dependent activation and nuclear translocation of a caspase-activated DNase, which results in internucleosomal DNA cleavage.[6] Apoptosis is dependent on the activation of a number of cysteine proteases belonging to the caspase family. However, caspases also are activated in settings not related to cell death, such as proinflammatory cytokine production.[7] There are several pathways to apoptosis-associated caspase activation. These include the extrinsic and the intrinsic pathways, designated based on the main origin of the first initiating signal, as well as the granzyme B-dependent pathway.[6] These pathways are summarized in Fig. 20.1. Caspase activation by the extrinsic (death receptor) pathway involves the binding of extracellular death ligands (FasL/CD95L, tumor necrosis factor-α [TNF-α], or TNF-related apoptosis-inducing ligand [TRAIL]) to various transmembrane death receptors.[8,9] Activation of these death receptors leads to the recruitment of adaptor proteins, such as the Fas-associated death domain protein (FADD), which in turn leads to the activation of the initiator caspase, caspase-8. Subsequently, caspase-8 activates the effector caspases, caspases-3 and -7, by proteolysis, leading to further caspase activation events that result in cell death. In the intrinsic (mitochondrial) pathway, caspase activation is initiated by intracellular stress or injury such as DNA damage, cytotoxic drugs, oxidative stress, or cytosolic Ca^{2+} overload among others and involves the permeabilization of the mitochondrial outer membranes.[7] Cellular stress activates one or more members of the BH3-only protein family and, above a certain threshold, overcomes the apoptosis-inhibitory members of the B-cell lymphoma-2 (Bcl-2) family. This leads to the assembly of Bax/Bak (Bcl-2-antagonist/killer-1–Bcl-2-associated X protein) oligomers within the mitochondrial outer membranes, which allow the release of intermembrane space proteins, such as cytochrome c, into the cytosol through the mitochondrial outer membrane permeability pore (MOMP). Cytochrome c, together with apoptotic protease-activating factor-1 (Apaf-1), deoxyadenosine triphosphate (dATP), and procaspase-9

TABLE 20.1

Differences in Morphologic and Biochemical Characteristics of Apoptosis and Necrosis

CHARACTERISTIC	APOPTOSIS	NECROSIS
Morphologic Characteristics		
Cellular swelling (swelling of cytoplasm and mitochondria)	Absent	Present
Plasma membrane integrity	Preserved	Lost
Membrane blebbing	Present	Absent
Chromatin	Discrete, condensed	Preserved
Nuclear condensation	Present	Absent
Cytosolic contents	Preserved	Released
Apoptotic bodies	Present	Absent
Phagocytosis	Present	Absent
Inflammatory response	Absent (to a large extent)	Present
Cell-cell adhesion	Lost early	Lost late
Pattern in tissues	Discrete, individual cells	Contiguous, groups of cells
Biochemical Characteristics		
Energy requirement	ATP dependent (active)	None (passive)
Adenosine triphosphate dependence	Yes	No
Caspase dependence	Yes	No
DNA cleavage	180 bp ladder pattern	Random, smear pattern
Cellular pH	Acidification	Unchanged
Mitochondrial permeability	Moderate loss	Severe loss
Mitochondrial potential	Transient loss	Permanent loss
Phosphatidylserine externalization	Yes	No
Propidium iodide uptake	No (except in late apoptosis)	Yes
High mobility group box 1 (HMGB1) release	No	Yes

DNA, Deoxyribonucleic acid.

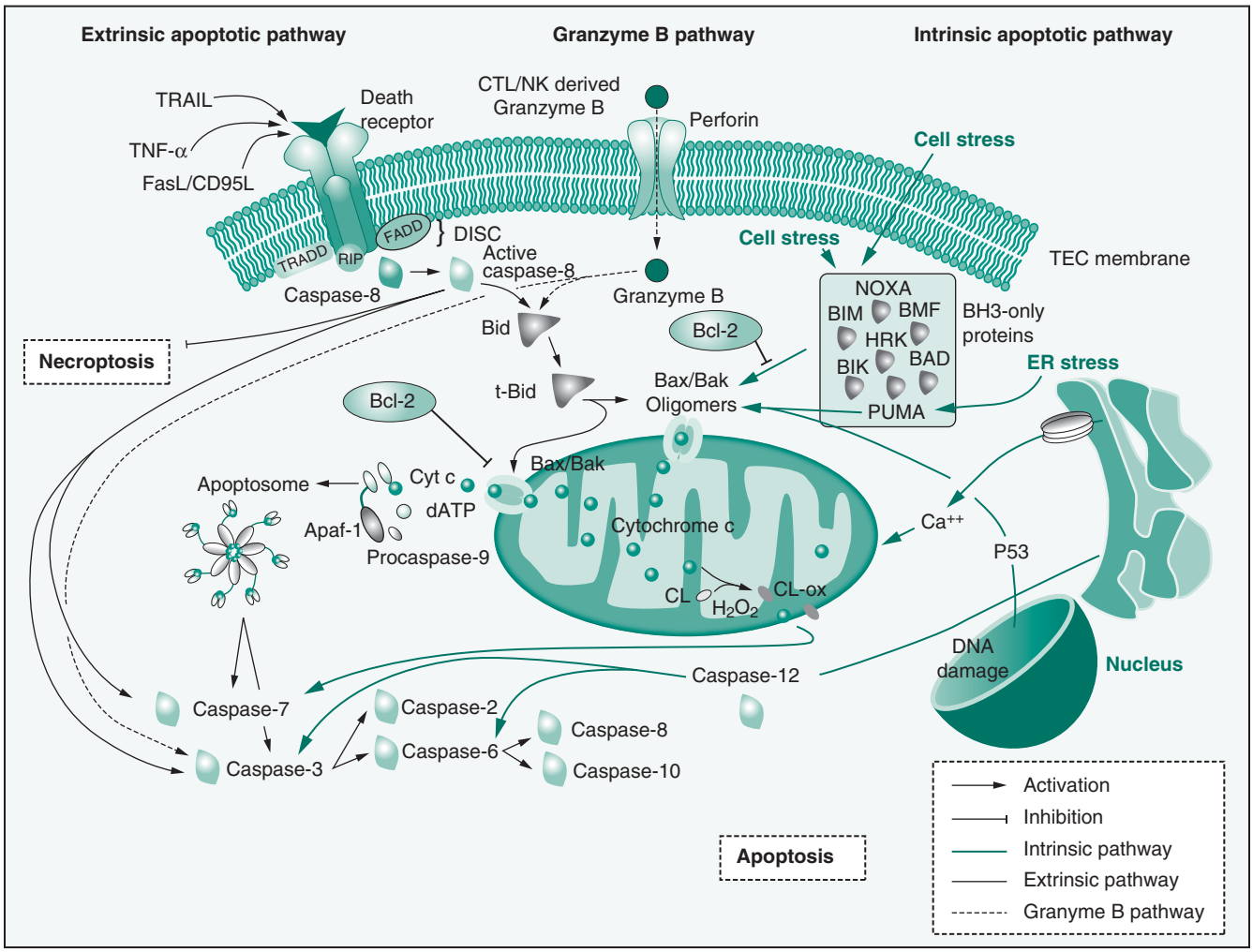

FIGURE 20.1 Major apoptotic pathways. The extrinsic pathway (black) requires activation of plasma membrane receptors such as Fas, type 1 tumor necrosis factor receptor (TNFR1), and TRAIL with subsequent signal transduction via adapter molecules such as Fas-associated death domain (FADD). This results in the activation of caspase-8 and formation of a death-inducing signaling complex (DISC), which propagates the death signal via three main pathways: directly through proteolytic activation of executioner caspases; by proteolysis of BCL2 homology-3 (BH3)-only protein BID, which induces the translocation of truncated BID (tBID) to mitochondrial membrane and provoke mitochondrial outer membrane permeabilization (MOMP); or by activation of the kinase RIP. In the intrinsic pathway (blue), cell stress results in activation of BH3-only proteins, which promote the oligomerization of Bax and Bak, producing channels that allow the release of components of the mitochondrial intermembrane space (such as Cytochrome c, Cyt c) and thus promoting MOMP. Translocation of cardiolipin (CL) to the outer mitochondrial membrane enables the interaction of CL with Cyt c. Binding of Cyt c to CL provokes the movement of the distal ligand of Cyt c (Met_{80}) away from its baseline position close to the heme iron. This enhances the access of small molecules such as H_2O_2 to the catalytic site of Cyt c, thus enabling it to oxidize CL. Oxidation of CL facilitates further detachment of Cyt c from mitochondrial membrane and thus its release, which is key in further signaling of the intrinsic apoptotic pathway. Released Cyt c binds Apaf-1, which recruits and activates caspase-9 via formation of the apoptosome. DNA damage induces stabilization of p53 tumor suppressor protein, which can result in transcriptional activation of BH-3–only proteins, and oligomerization of Bax/Bak. Cross-talk between these pathways is thus provided by two proapoptotic substances, Bcl-2 family protein BID and transcription factor p53. Bax translocation normally is prevented by cell survival promoter proteins Bcl-2 and Bcl-xL. Both caspases-8 and -9 activate caspase-3, which initiates the final morphologic cascades of apoptosis. The Granzyme B–dependent pathway involves the release of specialized granules from cytotoxic T lymphocytes or natural killer cells, and delivery to target cells. These granules contain perforins, pore-forming proteins that oligomerize and permit the entry of granzymes also contained in these granules. Once in the cytoplasm of the target cell, Granzyme B can induce apoptosis via cleavage of BID, or directly by cleavage of caspase-3 and -7. The *arrow* indicates activation, whereas the line with the flat end indicates inhibition. *CTL/NK,* cytotoxic T lymphocyte/natural killer); *dATP,* deoxyadenosine triphosphate; *RIP,* receptor interacting protein; *TRADD,* TNF receptor associated death domain; *TEC,* tubular epithelial cell.

form the apoptosome, a heptameric caspase-9–activating complex. Activated caspase-9 then propagates a proteolytic cascade of further effector caspase activation events and ultimate apoptotic cell death.There is potential for cross-talk between the extrinsic and intrinsic apoptotic pathways. Extrinsic death signals can cross-talk with the intrinsic pathway through the caspase-8–mediated proteolysis of the BH3-only protein, BID (BH3-interacting domain death agonist). Truncated BID (tBID) can then promote mitochondrial cytochrome c release and formation of the apoptosome.[6] In addition, the p53 tumor suppressor can modulate the extrinsic and intrinsic apoptotic pathways. The p53 gene is a potent transcription factor activated via DNA damage and cellular stress, which can regulate apoptosis by activating proapoptotic Bcl-2 family members as well as the Fas-dependent axis.[10–12] The granzyme B-dependent caspase activation route is a method by which cytotoxic T lymphocytes (CTL) or natural killer (NK) cells induce

apoptosis. It involves the delivery of granzyme B, a serine protease, into the target cell through CTL or NK cell granules, which contain numerous granzymes as well as a pore-forming protein, perforin that facilitates the entry of the granzymes. Granzyme B cleaves its substrates after aspartate residues and can process BID as well as caspases-3 and -7 to initiate apoptosis.

The downstream proteolytic targets of activated caspases include major cytoskeletal constituents (which lead to rounding of the cell and membrane blebbing in areas where the cytoskeleton has been weakened), nuclear envelope proteins such as lamins (which result in nuclear condensation and fragmentation), components of adhesion sites or complexes (which lead to cells retracting from neighboring cells and removal by phagocytes), proteins that function in transcription and translation, nucleases involved in DNA fragmentation such as caspase-activated DNase (CAD), substrates related to the fragmentation of the Golgi apparatus and disruption of mitochondrial function, and enzymes related to the generation of chemoattractants for phagocytes and translocation of the membrane phospholipid PS from the inner plasma membrane leaflet to the outer membrane leaflet.[6]

Regulated Necrosis

Necrosis is characterized by cytoplasmic granulation and cellular and organelle swelling (oncosis) and results in the rupture of the plasma membrane and organelle breakdown. It can lead to local inflammation secondary to the release of intracellular DAMPs and alarmins from dead cells, which are recognized by pattern recognition receptors (PRR) of the innate immune system. Necrosis has long been described as accidental and uncontrolled, a rapid cell death occurring as a consequence of extreme physical or chemical stress. However, it is now known that necrosis can exist as a highly regulated and genetically directed process. Over the past 10 years a number of regulated necrosis pathways have been described; the most well-studied is necroptosis.

Necroptosis

Necroptosis is the term used to describe a caspase-independent form of regulated necrosis that is dependent on receptor-interacting protein 1 (RIPK1) and RIPK3 activity.[2] It can be triggered by the ligation of death receptors *in the presence of caspase inhibition*, including TNF receptor 1 (TNFR1), TNFR2, FAS/CD95, TRAILR1 and TRAILR2, as well as by members of the PRR family, which are expressed by cells of the innate immune system to sense pathogen-associated molecular patterns (PAMPs).[13] Necroptosis requires the formation of the RIPK1/RIPK3/mixed lineage kinase domain-like (MLKL)–containing necrosome.[14] RIPK3 is activated by phosphorylation and, in turn, phosphorylates the pseudokinase MLKL, which has been shown to interact with membrane phospholipids and permeabilize the plasma membrane, leading to the release of DAMPs.[15] The same ligands (TNF-α, FasL, TRAIL) that can activate apoptosis also can activate necrosis. Cell death induced by the activation of death receptors thus may be executed through either apoptosis or necroptosis. The TNF signaling pathway is the most understood pathway leading to necroptosis and is a good example of this (Fig. 20.2).[15,16] Upon stimulation of TNFR1 by TNF, a conformational change occurs and allows the formation of the (TNFR1-associated death domain)–dependent receptor-bound complex I, which includes TNF receptor–associated death domain (TRADD), RIPK1, cellular inhibitor of apoptosis proteins (cIAPs), TNF receptor–associated factor-2 (TRAF2), and TRAF5.[13]

Ubiquitination of RIPK1 by cIAP recruits transforming growth factor-β activated kinase 1 (TAK1), TAK1-binding protein 2 (TAB2) and TAB3, which then initiate the canonical nuclear factor-κB (NF-κB) activation pathway. Conversely, RIPK1 can exert apoptotic or necroptotic cell death after deubiquitination by cylindromatosis (CYLD), A20 (TNFAIP3), cezanne (OTUD7B), or ubiquitin-specific peptidase 21 (USP21).[13,15] Deubiquitination allows RIPK1 to dissociate from the plasma membrane and interact with TRADD, Fas-associated death domain (FADD), procaspase-8, and Fas-associated death domain-like interleukin-1β-converting enzyme (FLICE)-like inhibitory proteins (FLIPs) to form a TRADD-dependent complex IIa, which allows activation of caspase-8, subsequent activation of the effector caspases, caspase-3 and -7, and leads to apoptosis.[17] The long isoform of FLIP ($FLIP_L$), which is upregulated by NF-κB, can form heterodimers with procaspase-8 that can cleave and inactivate RIPK1, RIPK3, and CYLD to prevent necroptosis. When caspase-8 is inhibited by caspase inhibitors or virally encoded proteins, phosphorylated RIPK1 and RIPK3 interact to form microfilament-like complexes called necrosomes.[18] CYLD deubiquitinates RIPK1 in the necrosome to facilitate its pronecrotic kinase activity.[19] The recruitment and phosphorylation of MLKL initiates necroptosis and results in JUN N-terminal kinase (JNK) activation, reactive oxygen species (ROS) production, and translocation of oligomerized MLKL to the plasma membrane, where it mediates calcium and/or sodium influx-dependent necroptosis.[20–22]

When cellular cIAPs are depleted and RIPK1 is not ubiquitinated, the formation of complex I leads to the upregulation of NF-κB–inducing kinase (NIK), activation of the noncanonical NF-κB pathway, and the formation of a large TRADD independent cytosolic complex comprising RIPK1, RIPK3, FADD, and the $FLIP_L$–caspase-8 heterodimer (RIPK1-dependent complex IIb or "ripoptosome").[15] Similar to complex IIa, RIPK1 and RIPK3 are cleaved and inactivated by caspase-8–$FLIP_L$ heterodimers, and the RIPK1-dependent complex IIb can induce apoptosis in the presence of caspase-8 activity or necroptosis when the function or recruitment of caspase-8 is defective.

Other Forms of Regulated Necrosis

Emerging pathways of specialized forms of regulated necrosis include cyclophilin D (CYPD)–mediated regulated necrosis involving the opening of the mitochondria permeability transition (MPT) pore, parthanatos, pyroptosis, ferroptosis, oxytosis, ETosis, NETosis, and pyronecrosis.[15] These processes are morphologically consistent with regulated necrosis but occur independently of RIPK1 or RIPK3 or occur in the presence of RIPK1 or RIPK3 inhibitors. They represent various forms of genetically controlled cell death that follow a period of oncosis with the various terms reflecting the process occurring in different physiologic conditions or cell types. The role that several of these pathways may have in renal injury is discussed later in this chapter.

CELL DEATH IN ACUTE KIDNEY INJURY

Experimental and human studies indicate that tubular epithelial cells can suffer one of two distinct fates after AKI. The majority of cells remain viable,[23–26] suggesting

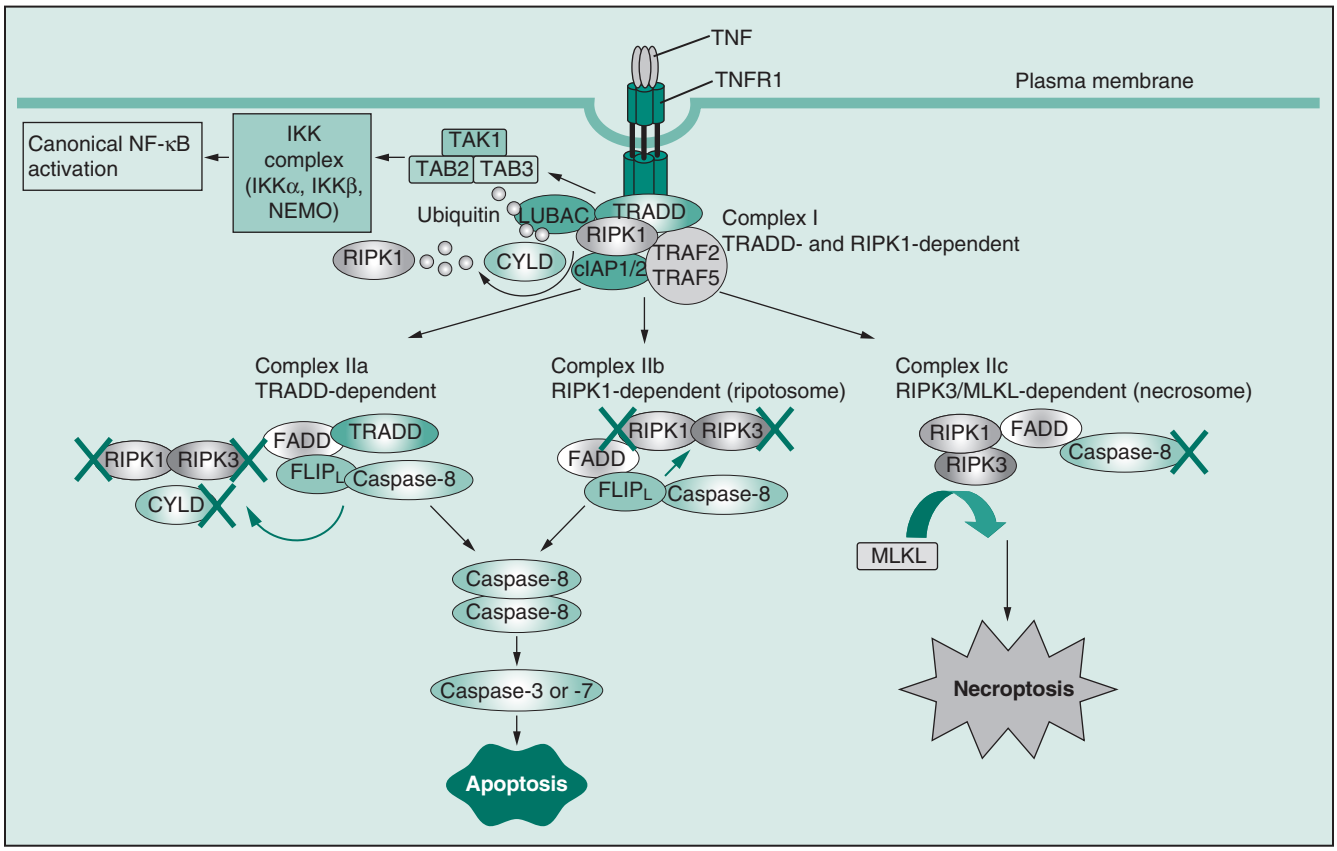

FIGURE 20.2 Tumor necrosis factor receptor (TNFR) activation. Tumor necrosis factor (TNF) binds to its receptor, TNFR1, and triggers the assembly of complex I (TNFR1, TNFR1-associated death domain [TRADD], receptor interacting serine/threonine-protein kinase 1 [RIPK1], TNFR-associated factor 2 [TRAF2], TRAF5, cellular inhibitor of apoptosis protein 1/2 [cIAP1/2], and linear ubiquitin chain assembly complex [LUBAC]). RIPK1 then undergoes Lys63-linked ubiquitylation by cIAP1/2 and linear ubiquitylation by LUBAC and leads to the docking of transforming growth factor-β-activated kinase 1 (TAK1) in complex with TAK1-binding protein 2 (TAB2), TAB3, and the inhibitor of NF-κB kinase (IKK) complex, and the subsequent activation of the canonical nuclear factor-κB (NF-κB) pathway. Cylindromatosis (CYLD) removes Lys63-linked polyubiquitins from RIPK1, allowing RIPK1 to dissociate from the plasma membrane and lead to the formation of the cytosolic TRADD-dependent complex IIa, where the interaction between TRADD, FAS-associated death domain (FADD), procaspase-8, and FLICE-like inhibitory proteins (FLIPs) leads to the activation of caspase-8, effector caspases 3/7, and apoptosis. Necroptosis is prevented by the heterodimeric caspase activity of the long isoform of FLIP ($FLIP_L$) and procaspase-8, which cleaves and inactivates RIPK1, RIPK3, and CYLD. Complex IIb is formed upon inhibition of cIAP1/2, TAK1, or NEMO. It is a TRADD-independent cytosolic complex consisting of RIPK1, RIPK3, FADD, and the $FLIP_L$-caspase-8 heterodimer often referred to as the RIPK1-dependent complex IIb or the "ripoptosome." Apoptosis is induced by the release of caspase-8, and the heterodimeric caspase activity of $FLIP_L$ and procaspase-8 again inhibits necroptosis by cleaving and inactivating RIPK1 and RIPK3. Complex IIc, also known as the necrosome, is formed when there is RIPK3 expression with concurrent decrease in expression or inhibition (by chemical caspase inhibitors or virally encoded proteins) of procaspase-8 and $FLIP_L$. The association and auto- and transphosphorylation of RIPK1 and RIPK3 leads to the recruitment of mixed lineage kinase domain-like protein (MLKL) by activated RIPK3. This leads to the formation of a supramolecular protein complex at the plasma membrane and subsequent necroptosis. *LUBAC,* linear ubiquitin chain assembly complex; *NEMO,* nuclear factor-κB essential modulator.

that they either entirely escape injury or are only sublethally injured and undergo recovery. A small proportion of tubular epithelial cells (TEC) display cell death in a patchy distribution, resulting most commonly from a combination of apoptotic and necrotic mechanisms. Indeed, in several models of AKI, necrosis and apoptosis are evident in varying proportions, mostly depending on the initiating cause rather than the anatomic location (apoptotic and necrotic cells are found in proximal and distal tubules, in the loop of Henle, and in the cortical and medullary regions).[27] For instance, regulated necrosis and apoptosis have been reported in ischemia reperfusion (IR), in nephrotoxic AKI secondary to cisplatin, and in sepsis. The contribution of these cell death mechanisms to renal functional impairment and recovery is equally variable depending on the cause of AKI. For example, RIPK3 knockout mice are protected from AKI after IR or administration of cisplatin. However, RIPK3/caspase-8 knockout did not confer further protection to animals subjected to IR but did to animals exposed to cisplatin, suggesting that extrinsic apoptosis may be significant to cisplatin nephrotoxicity but not to IR injury.[14] Importantly, the long-standing paradigm derived from animal models of IR that postulated necrosis as the pathophysiologic culprit of AKI has been challenged because the amount of necrosis that usually is observed during ischemic AKI is inconsistent with the level of dysfunction and fails to predict the development, need for support, or recovery from AKI.[28,29] This suggests that other mechanisms may be at play. The presence of apoptosis has been shown to better explain the renal dysfunction in animal models of ischemic AKI, where strategies targeting apoptosis result in protection of renal function.[30]

APOPTOSIS DURING ACUTE KIDNEY INJURY

Apoptosis is known to be a major mechanism of early tubule cell death in IR injury and nephrotoxic AKI.[27] Animal models of ischemic and nephrotoxic AKI as well as tissue biopsies in human AKI in the context of kidney transplantation consistently have shown apoptotic changes in tubular cells.[31–35] However, the extent of apoptosis in vivo is difficult to quantify for several reasons:

1. It is a rapidly occurring process.
2. It is heterogeneous in any individual kidney.
3. Clearance of apoptotic cells by the phagocytic machinery is known to be very efficient and fast.

Ischemia Reperfusion Injury

During ischemia, the proapoptotic protein Bax in the TEC is activated,[36,37] which results in reduction of the antiapoptotic Bcl-2,[38] and thus resetting of the pro-/antiapoptotic machinery (i.e., Bax/Bcl-2 ratio) balance towards the initiation of apoptosis. Although other possible Bcl-2 family members have been implicated, Bax/Bak double knockout cells and mice studies have provided evidence that Bax is the primary perpetrator of outer mitochondrial membrane damage.[39] Binding (and blocking) of antiapoptotic Bcl-2 proteins (Bcl-2 and Bcl-XL) by Bim and Bad also has been proposed as a mechanism favoring apoptosis in this setting. This is supported by the observation that activation of stress kinases, such as Akt and phosphorylates Bad, results in the release of Bcl-XL and thereby promotes cell survival.[40,41] Another important stress kinase activated in the setting of ischemia that is related to mitochondrial injury in the proximal TEC is glycogen synthase kinase 3-β (GSK3β). During ischemia and ATP depletion, GSK3β activation promotes apoptosis via Bax in TEC and in vivo, and inhibition by interference RNA knockdown promotes TEC survival.[42] Furthermore, pharmacologic inhibition of GSK3β (using TDZD-8) inhibited the activation of Bax and caspase-3, limited TEC damage, and protected renal function in a rat model of 30-minute IR after left nephrectomy.[42]

Other mechanisms have been proposed to explain the activation of the apoptotic machinery during ischemia in TEC. The first is that TEC expresses death receptors including Fas, TNFR1, and Fn14 receptor,[43] which are capable of inducing apoptosis via the extrinsic pathway. This is supported by the observation that blockade of Fas ameliorates renal injury after IR.[44] The second mechanism is mitochondrial fragmentation, a phenomenon that seems to occur before activation of Bax after ischemic injury. Mitochondrial fission (the process by which one mitochondria divides into two daughter organelles) is actually a normal quality control process that is balanced with fusion (the process of merging two mitochondria) to maintain a healthy pool of functional organelles. Fragmentation represents the disruption of these mitochondrial dynamics; fission is activated with the translocation of Drp1 to mitochondria in the context of cell stress and is accompanied by a characteristic arrest of fusion. Bak, which is located on the outer mitochondrial membrane and binds mitofusin-1 and -2 to maintain fusion, has been implicated in altering fusion during cell stress. Bak dissociates from mitofusin-2, binds with higher affinity to mitofusin-1 (arresting fusion),[45] and allows Drp1 to proceed with the formation of a restriction ring that activates the cleavage of the organelle. Blockade of Drp1, genetically or pharmacologically, prevents fragmentation, suppresses TEC apoptosis, and limits AKI.[46] A role for mitochondrial fragmentation has been established in in vivo rodent models of ischemia.[47] Another mitochondrial pathway of apoptosis is the oxidation of the mitochondria-specific phospholipid, cardiolipin, by the cytochrome c/cardiolipin complex.[48] A mitochondria-targeted compound SS-31, which is in clinical trials for heart failure, was reported to interact with cardiolipin and attenuate IR-induced TEC apoptosis in rats.[49] Finally, administration of exogenous growth factors such as epidermal growth factor, insulin-like growth factor, and vascular endothelial growth factor has been shown to decrease TEC apoptosis after ischemia.[40,50] The phosphatidyl-inositol 3-kinase/Akt pathway controls these protective effects by inhibiting the action of proapoptotic Bax.

Nephrotoxic Injury

Apoptotic cell death also has been demonstrated in nephrotoxic AKI. Cisplatin has been one of the most studied nephrotoxins given its widespread use as a chemotherapeutic agent for cancer. Cisplatin produces significant injury primarily in the S3 segment of the proximal tubule. It inhibits mitochondrial F1F0-ATPase, thereby limiting oxidative phosphorylation and altering mitochondrial membrane potential. This all precedes release of cytochrome c and the activation of the apoptotic machinery. Cisplatin also activates the p38 mitogen-activated protein kinase-mediated apoptotic pathway. These pathways function as an upstream signal for TNF-α mediated inflammation and injury, which has been identified as a significant mediator of tubular injury after exposure to cisplatin. Indeed, TNF-α–deficient mice are resistant to cisplatin toxicity,[51] although it remains unclear if TNF-α induces injury and alters renal function through inflammation or by directly inducing apoptosis. Cisplatin also alters the expression and activation of cell cycle proteins such as p21 and cyclin-dependent kinase 2 and increases the expression of the tumor suppressor, p53, which activates the executioner caspases-6 and -7 and results in apoptosis via the mitochondrial pathway.[52] Similarly to ischemic injury, cisplatin also activates Bax and Bak. The importance of this mechanism is underscored by the partial protection from cisplatin-mediated injury observed in Bax knockout mice.[53]

Rhabdomyolysis-induced myoglobin also can result in toxic injury to the kidney. In particular, myoglobin alters the interaction between the Jun N-terminal kinase and the 14-3-3 protein, both of which promote apoptosis through Bax, Bid, and Bad.[54]

CELL DEATH REGULATED BY NECROSIS DURING ACUTE KIDNEY INJURY

More recent evidence that necrosis occurs in regulated and genetically controlled pathways has led to a great expansion in our knowledge of some possible mechanisms behind the development of AKI. However, the exact mechanisms are still unknown, and much of the evidence is limited to in vitro or ex vivo studies.

Necroptosis

The contribution of necroptosis to ischemic, cisplatin, and hypoxia-induced injury in renal tubular cells was suggested

by the protective effect of necrostatin-1 (Nec-1), which is an inhibitor of RIPK1.[55-57] Nec-1 also protected rat proximal tubule cells in vitro from cyclosporine A–induced cytotoxicity.[58] RIPK3 (a downstream effector of RIPK1) knockout mice also were shown to be protected against IR renal injury.[14] It has been suggested that Nec-1 could prevent ferroptosis in RIPK1-deficient cells, a finding that questions the specificity of Nec-1 for necroptosis.[59] In fact, deletion of FADD or caspase-8 did not sensitize renal tubules to undergo necroptosis, and application of Nec-1 did not protect freshly isolated tubules from hypoxic injury.[60] In addition, in a murine model of contrast-mediated AKI, it has been suggested that although tubular cells did not appear to undergo cell death, Nec-1 also may alter peritubular perfusion.[61] The exact role of necroptosis in kidney injury remains unclear; however, these data suggest that necroptosis is most likely not the sole mode of regulated cell death in renal tubules.

Cyclophilin D–Mediated Regulated Necrosis

Necrotic cell death during acute ischemic injury is believed to largely occur secondary to mitochondrial permeability transition pore (MPTP) formation, which is thought to be composed of the F1/F0 ATP synthase on the inner mitochondrial membrane and Bax/Bak on the outer membrane.[62] The opening of the MPTP results in the movement of cytoplasmic water and solutes (≤1500Da) into the mitochondrial matrix, loss of the mitochondrial inner membrane potential, mitochondrial dysfunction, interference with ATP production, increased production of ROS, organelle swelling, catastrophic energy failure, and eventual cell rupture and death.[63] Cyclophilin D (CypD) is a regulator of the MPTP pore. CypD knockout mice are protected from the necrosis associated with IR injury in the kidney,[64] and the opening of the MPTP can be inhibited by the action of CypD inhibitors such as cyclosporin A. However, the MPTP can still open in response to high levels of ROS and calcium overload even in the absence of CypD.[65] IR to the parenchyma of the kidney leads to the activation of the nuclear repair enzyme, poly(ADP-ribose) polymerase 1 (PARP1), and the transcription factor p53, which can further initiate inflammatory signaling and pathways that induce necrosis through the opening of the MPTP.

Parthanatos

Parthanatos is a caspase-independent cell death mode involving the DNA damage-responsive enzymes, poly(ADP-ribose) polymerase (PARP) proteins, in particular PARP1. These are ADP-ribosyl transferase enzymes that transfer ADP-ribose groups from NAD+ to their target proteins and can control a variety of cellular processes. The activation of PARPs occurs through DNA breaks induced by ultraviolet light, ROS or alkylating agents, increased Ca^{2+} concentrations, or posttranslational modifications.[15,66,67] PARP activation contributes to the restoration of cellular homeostasis in conditions with mild DNA damage. However, PARP1 overactivation can deplete cells of NAD+ (impairs cellular metabolism) and consequently ATP (cellular energy crisis) and lead to the release and accumulation of the mitochondria-toxic PAR polymer from the nucleus, which then induces the translocation of active, truncated apoptosis-inducing factor (tAIF) from mitochondrial membrane to the nucleus and causes chromatin condensation and large-scale DNA fragmentation.[68,69] This culminates in a caspase-independent and ATP-independent form of cell death. The exact methods by which parthanatos is regulated remains unknown. PARP1 is proteolytically inactivated during apoptosis[70] but is likely involved in multiple pathways of regulated necrosis, although the extent of this remains elusive. A role for PARP1 activation was shown in TRAIL-induced necroptosis downstream of RIPK1 and RIPK3.[71] However, inhibition of PARP1 also has failed to prevent TNF-α–induced necrosis, and the inhibition of RIPK3 has failed to block PARP1 activation and cell death.[72] Pharmacologic inhibition or gene ablation of PARP1 protects rodent kidneys from both IR- and cisplatin-mediated AKI.[73,74] In ischemic kidneys, PARP1 expression and activity was increased in the S3 segments of the proximal tubule specifically, where it inhibited glycolysis via poly(ADP-ribosyl)ation of glyceraldehydes 3-phosphate dehydrogenase (GAPDH).[63] The decrease in activity of GAPDH leads to the vulnerability of the proximal tubule cell in the context of ischemic injury; the poly(ADP-ribosyl)ation of GAPDH and the resultant inhibition of anaerobic respiration exacerbates ATP depletion and induces necrosis.

Pyroptosis

The necrotic-like cell death mode, pyroptosis, is a form of programmed cell death that was once thought to occur solely in macrophages and leukocytes during inflammatory conditions in which intracellular danger signals lead to the production of proinflammatory cytokines, such as IL-1β and IL-18, and eventually lead to cellular swelling and death. Cells undergoing pyroptosis are characterized by the activation of nonapoptotic caspases such as caspase-1, which mediates cytokine maturation. It has been described in the renal TEC during animal and cellular models of IR and hypoxia-reoxygenation injury, respectively.[75] Membrane rupture and cytokine release into the interstitium lead to further inflammation and exacerbated AKI.

Ferroptosis

Ferroptosis is an iron-dependent cell death regulated by glutathione peroxidase 4 (GPX4).[76] Conditions of cysteine and reduced glutathione (GSH) depletion favor ferroptosis. Ferroptosis is characterized by accumulation of lipid oxidation products, particularly those produced from the oxidation of polyunsaturated fatty acids (PUFA) in membrane phospholipids.[60,76,77] As the name implies, there is a role of iron in ferroptosis, but the exact mechanisms of its involvement is not clear. Iron may act to catalyze free radical formation directly in the cytosol (e.g., via Fenton chemistry) and/or as a cofactor for an enzyme essential for the PUFA oxidation that precedes ferroptosis. The lipoxygenase (LOX) pathway has been implicated as a potential iron-dependent catalyst in phospholipid oxidation in ferroptosis.[76] Morphologic characteristics of ferroptosis include the presence of small mitochondria with increased membrane density. However, it is not associated with chromatin condensation, rupture of the plasma membrane, swelling of cytoplasmic organelles, or formation of cytoplasmic vesicles.[76] Recent studies provide evidence for the occurrence of iron-dependent ferroptosis in the renal tubules in models of severe IR and oxalate crystal-induced AKI.[60]

Among the eight GPXs that humans have, GPX4 is the only one that can reduce oxidized phospholipids in membranes, thus its catalytic activity is critically important for cellular

health. Conditional deficiency of GPX4 in the kidney has been shown to cause ferroptotic death of tubular epithelial cells leading to acute kidney injury, which was ameliorated by liproxstatin-1, a spiroquinoxalinamine derivative.[59] GPX4 activity relies on GSH levels, and GSH depletion leads to the loss of function of GPX4.[78] The key determinant of GSH synthesis in the cell is the availability of cysteine, which is transported into the cell as cystine (the oxidized form of cysteine) via the system Xc (cysteine/glutamate antiporter). Inhibitors of system Xc (such as Erastin) and inhibitors of GPX4 (such as RSL3) can trigger ferroptosis.[76,77]

Cell death caused by inhibition of the Xc– Cys/Glu antiporter by an excess of the neurotransmitter glutamate originally was classified as oxytosis.[15,79,80] In oxytosis, excess extracellular glutamate is thought to inhibit system Xc, thus decreasing intracellular cysteine and subsequently GSH levels. It is likely that oxytosis and ferroptosis share common pathways at least in some tissues. However, oxytosis is known to heavily rely on calcium influx, which activates the noncaspase proteases, calpains, facilitates lysosomal membrane permeabilization (LMP), and induces regulated necrosis,[15,81] whereas ferroptosis seems to have iron-dependent rather than calcium-dependent signaling.[76] In line with this ferroptosis can be inhibited by the iron chelator, deferoxamine, which scavenges accumulated iron in lysosomes to inhibit Fenton-type reactions.[76] Ferroptosis also can be inhibited pharmacologically by ferrostatin-1 (Fer-1), a synthetic, potent antioxidant molecule whose mechanism of action is still not entirely clear.[76,82] A third-generation ferrostatin, 16-86, was shown to decrease renal injury after severe IR.[60]

POTENTIAL THERAPIES

Although apoptosis has been the target for drug development for many years, the recent advancement in knowledge of the cellular machinery behind additional nonapoptotic regulated cell death pathways has led to the development of several promising strategies for new therapeutics (summarized in Table 20.2).

TABLE 20.2

Promising Pharmacologic Inhibitors of Regulated Cell Death

COMPOUND	MOLECULAR TARGET	PRIMARY EFFECT	INDICATION(S) AND CURRENT RESEARCH/ DEVELOPMENT STATUS
IDN-6556	Caspases	Small-molecule, pan-caspase inhibitor	Liver transplant (phase 2 clinical trials) Hepatitis C (phase 2)
IDN-6734	Caspases	Small-molecule, pan-caspase inhibitor	Acute myocardial infarction (phase 1)
Amifostine	P53	Small molecule; inhibits p53	Reduction of cisplatin nephrotoxicity (FDA approved)
Minocycline	Cytochrome c release	Small compound; inhibits cytochrome c release	Amyotrophic lateral sclerosis (phase 3) Huntington disease (phase 2)
SS-31 (elamipretide)	Cardiolipin	Inhibits cytochrome c peroxidase activity, protects mitochondrial cristae membranes from damage	Heart failure (phase 2), primary mitochondrial disease (phase 2), atherosclerotic renal artery stenosis/IR injury (phase 1 + 2)
Necrostatins, ponatinib[83]	RIPK1	Stabilize RIPK1	Ponatinib is approved for adults with T3 15I-positive chronic accelerated or blast-phase chronic myeloid leukemia, or Philadelphia chromosome positive ALL[84]
GSK872, GSK840, dabrafenib[85,86]	RIPK3	Blocks phosphorylation of MLKL	Dabrafenib, BRAF-mutant tumors including melanoma (phase 1, 2, and 3)
Necrosulfonamide[87]	pMLKL	Unknown	Experimental preclinical studies
Sanglifehrin A, cyclosporin A[14]	Cyclophilin D	Prevents mitochondrial membrane potential-related necrosis	Sanglifehrin and other cyclophillin inhibitors in hepatitis C
INO-1001	PARP	Small molecule; inhibits PARP (poly [adenosine diphosphate–ribose] polymerase)	Ischemia-reperfusion injury (phase 1)
Olaparib[87]	PARP1	Inhibits PARP1-mediated parthanatos	Advanced solid tumors including pancreatic, breast and lung cancer (Phase I/II)
GPX4 mimics	GPX4	Blocks ferroptosis	Experimental preclinical studies
Ferrostatins[82] Liproxstatin-1[59]	Lipid peroxidation	Loss of plasma membrane integrity	Preclinical studies
Adalimumab	TNF-α	Anti–TNF-α neutralizing monoclonal antibody	Rheumatoid arthritis, psoriasis, Crohn's disease (FDA approved)
Infliximab	TNF-α	Anti–TNF-α neutralizing monoclonal antibody	Rheumatoid arthritis, Crohn's disease (FDA approved)
Etanercept	Type 2 TNF receptor	Type 2 TNF receptor (TNF-R2)/ immunoglobulin G fusion protein, inhibits TNF-α	Rheumatoid arthritis, Crohn's disease (FDA approved)
Edaravone	Free radicals	Small molecule; free radical scavenger; antioxidant	Acute myocardial infarction (phase 3) Approved in Japan for stroke

FDA, US Food and Drug Administration; *RIPK1,* receptor interacting serine/threonine-protein kinase 1; *MLKL,* mixed lineage kinase domain-like; *TNF,* tumor necrosis factor.

Modified from Mulay et al. Necroinflammation in kidney disease. *J Am Soc Nephrol* 2016;27:27–39; Xie et al. Ferroptosis: process and function. *Cell Death and Differentiation* 2016;23:369–379; ClinicalTrials.gov.

Key Points

1. Cell death is a normal process that occurs in health and disease and can be classified into two distinct categories: accidental cell death and regulated cell death.
2. Regulated cell death, particularly the subtype programmed cell death, includes a number of genetically encoded pathways that are used by cells to maintain homeostasis in the face of adversity and thus represents important adaptive strategies.
3. Apoptosis and regulated necrosis have been documented in AKI secondary to ischemia and ischemia reperfusion and nephrotoxic injury.
4. Regulated cell death is considered an integral component of the pathophysiologic mechanism leading to AKI of diverse causes such as ischemia, ischemia reperfusion, and nephrotoxicity.
5. Multiple regulated cell death pathways have now been identified, and the understanding of these mechanisms may provide insight into novel targets for the development of mechanism-based therapies to prevent and treat acute kidney injury.

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