Chapter 1

From Apoptosis to Regulated Necrosis: An Evolving Understanding of Acute Kidney Injury

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http://dx.doi.org/10.5772/intechopen.74816

Abstract

Acute kidney injury (AKI) is a prevailing health threat around the world with high mortalities and heavy economic burdens. In the past, apoptosis was recognized as the main contributor to the pathogenesis of AKI. However, recent evidence has suggested that regulated necrosis also plays an important role in the pathologic process of various types of renal damages, improving our limited understanding of the complex mechanisms underlying AKI. Regulated necrosis is a newly identified type of "programed cell death" with morphologic features of necrosis, which includes necroptosis, ferroptosis, parthanatos, pyroptosis, etc. In this chapter, we summarized the molecular pathways of both apoptosis and regulated necrosis, and reviewed the potential roles and corresponding mechanisms of various cell deaths in AKI based on recent advances. We also discussed the therapeutic potentials and clinical implications based on manipulating regulated cell death. Taken together, the progress in this field lays the ground for better prevention and management of AKI in the future.

Keywords: apoptosis, regulated necrosis, necroptosis, acute kidney injury

1. Introduction

The balance between cell survival and cell death laid the foundation for any individual organisms. Cell death, especially molecularly regulated cell death, has been extensively investigated for decades in life science and medicine. Historically, cell death was roughly classified as different types: apoptosis and necrosis [1]. The term "apoptosis" derives from ancient Greek and refers to the developmentally programmed and molecularly controlled cell death, with featured morphologic changes including cell shrinkage, nuclear and cytoplasmic condensation,

DNA fragmentation, and the formation of apoptosomes [2]. Necrosis, differently, was well recognized as an accidental cell death, with unique characteristics consisting of cellular swelling, breakdown of plasma membrane integrity, and release of intracellular contents, all of which are absolutely distinct from programmed cell death [3].

Caspase-dependent apoptosis is the first identified programmed form of cell death and was regarded as the only form of regulated cell death for a very long time. However, this restricted view has been challenged with the findings of new types of regulated cell death. Exciting breakthroughs in recent years have identified a group of novel forms of cell death, with morphologic features of necrosis but molecularly controlled, termed regulated necrosis [4]. According to the recommendations of Nomenclature Committee on Cell Death (NCCD), the types of regulated necrosis are composed of necroptosis, mitochondrial permeability transition (MPT)-mediated regulated necrosis (MPT-RN), parthanatos, ferroptosis, and pyroptosis [5]. Nowadays, it is well documented that regulated necrosis broadly participates in various biological processes including organism development, immune defenses and various pathophysiological processes [4, 6]. An accumulating body of evidence has demonstrated that regulated necrosis contributes to the pathogenesis of numerous diseases and damages in different organs or tissues [4, 6, 7].

Acute kidney injury (AKI), with a heavy health burden globally, still remains a severe condition in daily clinical routines to date [8]. It is estimated that every year approximately 13.3 million people are diagnosed with AKI and AKI contributes to about 1.7 million deaths around the world per year [9]. In the past, apoptosis was supposed as the predominant form of cell demise that is responsible for renal dysfunction during AKI [10]. Much study in recent years have indicated that programmed form of cell death, no matter apoptosis or regulated necrosis, plays an important role in keeping kidney tissue homeostasis as well as contributing to the pathogenesis of AKI [11, 12]. Different methods targeting the apoptotic molecular signals have been widely explored for AKI treatment. In spite of the solid protective effects of these treatments observed in animal models, the experimental anti-apoptosis intervention strategies cannot still be translated into medical practice, which might be, at least partially, due to our ignorance of regulated necrosis in the process of AKI. An improved understanding of the pathogenesis of AKI under the view of regulated cell death might provide potential therapeutic regimens based on manipulating both apoptotic and regulated necrotic pathways.

Therefore, in this chapter, we summarized the molecular pathways of both apoptosis and regulated necrosis, and reviewed the potential roles and corresponding mechanisms of various cell deaths in AKI based on recent advances. We further discussed the therapeutic potentials and clinical implications from the clinician's underground.

2. Apoptosis in AKI

2.1. Signaling pathways of apoptosis

The classic apoptosis can be activated by the intrinsic and extrinsic pathways that both rely on the involvements of caspases [13]. To date, the signaling pathways of apoptosis

have been well delineated and for the integrity of the present review, we only emphasize on the basic and major signal events of classic apoptosis. The mitochondria are the crucial converging site for the intrinsic and extrinsic signaling [14]. And specifically, mitochondrial outer membrane permeabilization (MOMP) serves as the key to initiate the final steps of apoptosis. MOMP results in the release of pro-apoptotic components of mitochondria, including cytochrome c, AIF, etc., and thereby the activation of subsequent apoptotic executive mediators [15]. MOMP can be regulated by BCL2 family proteins consisting of pro- and anti-apoptotic factors [10]. Bax and Bak are the main members of BCL2 family and serve as promoters for MOMP; Bcl-2 and Bcl-XL, in contrast, play just the opposite role. Notably, the balance between the two parts determines the fates of cells. In the intrinsic pathway, several cellular stresses including DNA damage, intracellular calcium overload and growth factor ablation can directly induce MOMP. In response to the release of cytochrome c, apoptosome consisting of caspase 9, Apaf-1 and cytochrome c is formed in plasma, which initiates the execution of apoptosis. In the extrinsic pathway, ligation of death receptors results in the formation of DISC, which recruits adapter proteins subsequently leading to combination of caspase-8/FLIP heterodimer and caspase-8 homodimer. The homodimer then cleaves effector proteins caspases-3, -6 and -7 and thereby finishes the execution of apoptosis [16]. It is worthy to note that the extrinsic pathway is able to amplify the apoptotic signals by initiating the intrinsic pathway through caspase-8-independent activation of tBid, which can cause MOMP [14].

2.2. The contribution of apoptosis to AKI

As early as 1992, Schumer et al. have demonstrated the first evidence for the existence of apoptosis in AKI with morphologic, biochemical, and molecular detection methods [17]. Thereafter, studies about the role of apoptosis AKI and relevant potential therapeutic modalities have been under intensive investigations. Overwhelming reports exhibited that apoptosis is functionally relevant to various kinds of AKI, of which ischemia–reperfusion injury (IRI) is the most well documented [18, 19]. Besides, sepsis is another common cause of AKI, especially in the intensive care units. Studies demonstrated that apoptosis serves as an important foundation for the pathogenesis of sepsis-related AKI [20–22]. In addition to IRI and septic renal injury, toxic kidney injury is also a common clinical condition contributing to a high rate of morbidity and mortality. Cisplatin, for instance, is a widely-used chemotherapy drug with relatively high nephrotoxicity. As supposed, apoptosis plays a vital role in the pathologic process of toxic renal injury [23].

In the context of AKI, apoptotic cells are detected in different types of renal tissues within both cortical and medullary regions. The most common sites are the renal tubules, especially the proximal tubules [10]. Besides, AKI can also cause apoptosis in other renal cells, for example the vascular endothelial cells which in turn deteriorate kidney damages [24, 25].

Collectively, these studies proved the widely occurrence and definitely great functional contributions of renal apoptosis during AKI. The apoptosis was supposed as the most prominent cell death in AKI for nearly two decades, until increasing evidence shows regulated necrosis is to great extent responsible for the pathogenesis of AKI as well.

3. Necroptosis in AKI

3.1. The signaling pathways of necroptosis

Necrostatin-1 (Nec-1) was identified as a specific inhibitor of receptor-interacting protein kinase 1 (RIP1) in 2008. Since then, the molecular pathways of necroptosis have been extensively studied [26, 27]. As the best-characterized regulated necrosis, necroptosis is shown to be initiated by the engagement of death receptors, Toll-like receptors (TLRs), interferon signals, as well as intracellular stimuli from protein DNA-dependent activator of IFN regulatory factors (DAI) [28]. The details about the signaling of necroptotic cell death have been already reviewed [7, 28], and in this character, we only present the molecular pathways of TNF-α-induced necroptosis in the absence of functional caspases. Upon the binding of TNF-α to TNF receptor (TNFR)1, the adaptor molecules Fas-associated death domain (FADD) and TNF-receptor-associated death domain (TRADD) are recruited to the ligated TNFR1 successively. These adaptor molecules then bind to RIP1. Subsequently, RIP1 combines with RIP3 to assemble a complex termed "necrosome" via the interaction of RIP homotypic interaction motif (RHIM) domain on both RIP1 and RIP3 [29–32]. RIP3 goes through autophosphorylation within necrosome, which leads to the activation of RIP3 [30, 31]. Activated RIP3 subsequently recruits and phosphorylates the downstream MLKL, which is believed as the executor of necroptosis [33, 34]. The exact mechanisms underlying the execution activity of MLKL are not totally delineated yet. It is thought that phosphorylated MLKL goes through a molecular switch to translocate to the membrane and consequently disrupt the integrity of plasma membrane to finish the action of necroptosis [35, 36]. In addition, study showed that MLKL could also induce mitochondria fission via the action of phosphoglycerate mutase family member 5 (PGAM5) and dynamin-related protein 1 (Drp-1) [37]. As mitochondria play an important role in apoptosis, this result suggests a broad involvement of mitochondria in different types of cell death. But the relative contribution of mitochondria-mediated damages in the background of necroptosis needs further confirmation [38]. It is notable that other necroptotic pathways mediated by TLRs, interferon signals and DAI converge on the RIP3 and share the same downstream executing pathway, indicating the indispensable role of MLKL in necroptosis.

3.2. The contribution of necroptosis to AKI

In 2012, Linkermann and colleagues found the protective effect of Nec-1, the first-generation of necrostatins, in a murine model of renal IRI, providing the first evidence of the presence of necroptosis in AKI [39]. In this study, Nec-1 was shown to prevent ischemic kidneys from renal dysfunction and tissue damage, indicating both functional and histological relevance of necroptosis in the pathogenesis of AKI. The pan-caspase inhibitor zVAD that was used to inhibit apoptosis in treatment of kidney diseases, surprisingly, was demonstrated non-protective in the same research. The reasons of this conflict result compared with a previous report that demonstrated the protective effect of zVAD in the context of IRI need further investigations [40]. The different methods adopted in these two independent researches may partially explain the incontinence of the therapeutic effects of zVAD. First, different clamping

time of the renal pedicles in these studies might result in the alterations of the magnitude of apoptotic and necroptotic cell death, which subsequently leads to a changed treatment effect. In addition, the time courses of the zVAD administration were also different. The profiles of cellular death kidneys will continuously evolve during AKI and apoptosis does not occur immediately after the onset of ischemia [10]. Therefore, application of zVAD just 15 min before ischemia might diminish its therapeutic effect. In accordance with this research, the protection of Nec-1 in rat and human renal tubular epithelia cells (TECs) against ischemic insults was confirmed by other investigators *in vitro* [41, 42]. Importantly, more convincing evidence of necroptosis in renal IRI was provided by using the *Rip3*-knockout mice in the following study. Linkermann et al. exhibited that *Rip3*-knockout mice improved kidney damages in contrast to wild-type mice, and Nec-1 administration in *Rip3*-knockout mice could not lead to addictive protection [43].

Besides IRI, accumulating evidence demonstrated necroptosis also contributes to AKI induced by nephrotoxic agents, including cisplatin, cyclosporin A (CsA) and imaging contrast. Tristao et al. found that Nec-1 can provide additional protection against the cisplatin-associated damage on human renal TECs on the basis of zVAD treatment [44]. Later, Linkermann [43] and Xu [45] further provided more reliable evidence to confirm the role of necroptosis in cisplatin-induced AKI with *Rip3*- and *Mlkl*-knockout mice models respectively. Ouyang et al. showed that rat TECs subjected to CsA, a widely used immunosuppressive drug for organ transplantation and other autoimmune diseases, were effectively protected by Nec-1 and knockdown of *Rip3*, indicating a role of necroptosis in the pathologic process of CsA-related AKI [46]. Furthermore, Nec-1 was similarly shown to prevent from contrast-induced AKI in a following study [47].

The existence of necroptosis was also indicated in a glycerol-induced rhabdomyolysis model [48]. The authors found that treatment with Nec-1 led to a reduced tubular necrosis, underscoring the importance of TNF-alpha-mediated tubular necroptosis in this model [48].

Taken together, these findings demonstrated that necroptosis is of vital importance for the pathogenesis of various types of AKI, suggesting a potential therapeutic checkpoint which invite further investigations basically and clinically.

4. Other regulated necrosis in AKI

4.1. MPT-RN

Mitochondrial permeability transition (MPT) mediated regulated necrosis (MPT-RN) is featured by the opening of a trans-mitochondrial membrane pore, namely the MPT pore (MPTP) [49]. CypD is identified as a controller of MPTP, which promotes the opening of this channel [50]. CypD interacts with another regulator the F_0F_1ATP synthase that maintains the inactivation of MPTP. Although the upstream pathways that initiate MPT-RN and the exact mechanisms to modulate the activity of CypD and F0F1ATP synthase remain elusive, it is believed that the opening of MPTP is capable to result in translocation of NAD^+ to cytosol and mitochondrial potential disruption [51]. NAD^+ along with ATP can be further consumed in the

process of NAD^+ glycohydrolases. The final result of these physiopathological alterations is the occurrence of regulated necrosis. Therapeutically, MPT-RN can be inhibited by sanglifehrin A and cyclosporin A [52].

Several independent groups of investigators have demonstrated the role of MPT-RN in AKI by detecting the contribution of CypD in the pathogenesis of kidney injuries. In 2009, Devalaraja-Narashimha et al. found that renal function, as well as the magnitude of erythrocyte trapping, tubular cell necrosis, tubular dilatation, and neutrophil infiltration in kidney histology improved significantly in CypD-deficient mice in the background of renal ischemia–reperfusion injury compared with wild-type mice [53]. Later, Hu et al. showed that knockdown of CypD by RNA interference could also protects rats from renal IRI [54]. The protective effects of CypD inhibition against kidney IRI *in vitro* and *in vivo* were further confirmed by Park et al. using a mouse model null for *Ppif*, the gene encoding CyPD [55]. Linkermann et al. evaluated CypD-deficient mice and RIP3-deficient mice in renal IRI and found that RIP3 deletion seemed to offer a better protection, providing a direct comparison between the selective contributions of MPT-RN and necroptosis to renal IRI [43]. More importantly, the researchers also showed CypD-RIPK3 double-knockout or combined application of Nec-1 and sanglifehrin A were more protective than inhibiting either of these two genes alone, indicating the coexistence of independent regulated necrosis in the same physiopathologic process.

4.2. Parthanatos

Parthanatos is the poly(ADP-ribose) polymerase 1 (PARP1)-dependent regulated necrosis [56]. PARPs cause the poly(ADP-ribosyl)ation (PARylation) of target proteins and thereby regulate various cellular bioactivities [57]. Different stimuli such as DNA breaks and Ca^{2+} signaling can activate PARP1, which induces the accumulation of PAR polymers. Both PARP1 and PAR polymers are able to delete NAD^+ and ATP via their PARylation [58, 59].

Increasing body of researches have demonstrated that parthanatos plays an important role in the pathogenesis of various types of AKI. By using genetic knockout models or chemical inhibitors of PARP1, several studies provided direct evidence that PARP1-dependent parthanatos was functionally related to renal IRI and showed that inhibition of PARP1 could effectively improve renal injuries [60–62]. Besides *in vivo* models, upregulated PARP1 were also detected in cultured renal tubular epithelial cells that were subjected to H_2O_2 [60, 63]. In addition to renal ischemic injury, parthanatos also contributes to AKI induced by various nephrotoxic agents [64–66]. Furthermore, the contribution of parthanatos to AKI was determined in a LPS-induced sepsis-related kidney injury model [67–69]. Taken together, these studies exhibit that parthanatos is an important participant in different forms of AKI, indicating a promising therapeutic target in clinical routines.

4.3. Ferroptosis

Ferroptosis was discovered during a pharmacological intervention in highly resistant RAS-transformed tumor cells with application of erastin by Dixon et al. [70]. Erastin, a lethal small molecule, was originally screened to eliminate cancer cells and was found to cause an unrecognized

type cell death that were distinct from either apoptosis, necroptosis, or other known regulated necrosis. This form of cell death, characterized by perioxidation, relies on accessible intracellular iron, and is therefore named as ferroptosis (ferro, ferrous ion) [70]. Erastin is believed to inhibit the system Xc- cystine/glutamate antiporter, which plays a key role in the exchange of extracellular cystine and intracellular glutamate. Cystine is required for synthesizing glutathione (GSH). Glutathione peroxidase 4 (GPX4) is an indispensable enzyme maintaining intracellular homeostasis by prevent reactive oxygen species accumulation and lipid peroxidation. Importantly, GPX4 is determined as a key inhibitor of ferroptosis, and its function is dependent on intracellular levels of GSH [71]. Therefore, inhibition of the system Xc- cystine/glutamate antiporter could result in a catastrophic decrease of GSH and thereby functional ablation of GPX4. More details about the emerging signaling of ferroptosis are provided by Yang and Stockwell [72]. Upon the introduction of ferroptosis, Dixon and Stockwell also identified a small molecule—ferrostatin-1—as an inhibitor of ferroptosis, namely the first-generation of ferrostatins, which serves as a crucial tool in the research of ferroptosis thereafter [70]. Due to the pharmacological instability of ferrostatin-1, second- and third-generation ferrostatins have been developed with promising therapeutic outlooks.

In 2014, three different teams from German and the United States reported that ferroptosis served as a crucial participant in the pathologic process of renal injuries. Friedmann Angeli and colleagues used inducible Gpx4 knockout mice to exhibit that deletion of Gpx4 led to ferroptosis-related acute renal failure and associated death. They also confirmed that Gpx4 ablation could cause extra-mitochondrial lipid peroxidation which thereby triggered ferroptosis. Furthermore, Liproxstatin-1, a spiroquinoxalinamine derivative, was demonstrated to inhibit ferroptosis *in vitro* and *in vivo* [73]. Rhabdomyolysis, as a severe and common clinical condition, is regarded as one of the main causes of AKI and rhabdomyolysis-induced AKI accounts for ~10% of all AKI cases. Rhabdomyolysis is the disruption of striped muscle followed by massive releases of intracellular molecules, in particular electrolytes and myoglobin, which induces oxidative damages and cell death. Skouta et al. subjected freshly isolated mouse kidney proximal tubules to an *ex vivo* model of rhabdomyolysis-induced AKI with or without classic ferroptosis inhibitor Ferrostatin-1 and found that Ferrostatin-1 could effectively prevent cell death [74]. Linkermann et al. have found that ferroptosis contributed to the synchronized necrosis of freshly isolated renal tubules in the context of IRI and oxalate crystal-induced acute kidney injury and Ferrostatin-1could alleviate the synchronized necrosis. Linkermann and colleagues also developed a third generation ferrostatin 16–86 with a more stable biochemical and metabolic feature, which were able to protect mice from severe IRI [75]. These reports provide direct evidence for the vital importance of ferroptosis in the pathogenesis of several types of AKI, indicating a potential therapeutic checkpoint in treating renal diseases.

4.4. Pyroptosis

Pyroptosis was initially referred to a certain kind of highly inflammatory cell death of infected macrophages [76]. Later, the cellular profile of pyroptosis has expanded from macrophages to other cell types. It is notable that a distinct feature of pyroptosis is the active release of IL-1b and IL-18 during pyroptotic cell death process, which contributes greatly to the high immunogenicity of pyroptosis [77]. Although the signaling pathway of pyroptosis, especially

the execution mechanisms, still remains elusive now, it has been documented that pyroptotic cell death results from caspase 1-dependent formation of transmembrane channels and subsequent osmotic pressure disruption [78]. In addition to caspase 1, caspase 11 are further identified as another crucial mediator of pyroptosis [79, 80]. Pyroptosis can be suppressed by chemical inhibitors VX-740, VX-765 as well as virus-derived molecule cytokine response modifier A (CrmA) [4, 76]. Few researches have focused on pyroptosis in the context of AKI. Yang et al. found that the expressions of pyroptosis-associated markers caspase 1 and caspase 11 were both significantly upregulated in a rat model of IRI and pyroptosis could also be observed in an *in vitro* model of hypoxia-reoxygenation, suggesting the existence of pyroptosis in kidney IRI [81]. Additionally, the authors demonstrated a possible regulation of endoplasmic reticulum (ER) stress on pyroptosis. But this interesting report provided no direct evidence for the functional responsibility of pyroptosis in renal injuries. The underlying physiological and pathological relevance of pyroptosis in kidneys, therefore, still remains unclear and needs intensive investigations urgently in the future [82].

5. Therapeutic implications

5.1. Combination therapy

Anti-apoptosis-based therapeutic strategies have been intensively explored for the treatment of AKI prior to the recognition of regulated necrosis. However, few anti-apoptosis interventions have been widely applied in clinical practice, despite the promising results obtained in animal models, which might be, at least partially, ascribed to our limited understanding of the regulated cell death in the context of AKI.

Thanks to the improved interpretation of the roles of regulated necrosis in kidney diseases, it is now possible to manipulate the apoptotic and regulated necrotic signaling simultaneously. In an interesting study, Tristao et al. found that the combined use of apoptosis and necroptosis inhibitors could provide additional protection in AKI, suggesting that the combination therapy targeting apoptosis and regulated necrosis might provide optimized therapeutic alternatives [44]. Moreover, combined inhibition of different regulated necrosis is also effective. Linkermann et al. demonstrated that the third-generation ferroptosis inhibitor 16–86 was able to further enhance the protective effect on IRI via combined treatment with necrostatins and MPT-RN inhibitor [75]. These results indicate that the combined blocking of several different regulated cell deaths hold the great promise to improve the current treatment for AKI. But further investigations are still needed.

5.2. Screening for next-generation inhibitors

Searching for chemical inhibitors or generating novel compounds targeting the critical checkpoints makes it possible to manipulate regulated cell death more efficiently. Compared with apoptosis whose inhibitors have been widely explored, the inhibitors of regulated necrosis warrant more explorations in the future. For example, Nec-1 is the first-generation of necrostatins that initially identified as the RIP1 inhibitor, and later widely used as a tool to distinguish necroptosis. However, a recent study demonstrated that Nec-1 was not a specific inhibitor against necroptosis

because Nec-1 could also protect *Rip1*$^{-/-}$ cells from ferroptosis, indicating an inhibitory effect of Nec-1 on ferroptosis [73]. More seriously, unexpected side effects of Nec-1 were observed on renal peritubular diameters [47], and on the action of indolamin-2, 3-dioxygenase (IDO) [83]. Besides, a relatively short half-life period of Nec-1 also hampers its final clinical application [83]. Thus, Nec-1 is a typical example of the original edition of regulated necrosis inhibitors that are prevailingly nonspecific and pharmacologically unstable. Great efforts have been made to searching for more effective and reliable inhibitors and a series of new inhibitors have been reported recently [34, 73, 75, 83–91]. It is remarkable that some researchers performed screens in the FDA-approved agent pools to identify effective drugs to suppress necroptosis, providing a helpful screening strategy [84, 87]. Considering that FDA-approved drugs have already been carefully evaluated in critical procedures before the clinical application, their pharmacological features and side effects are well documented. Most "new" inhibitors have not been extensively evaluated and therefore need elaborate investigations in the near future. It is indeed exciting that increasing agents targeting regulated cell death have entered clinical trials for the treatment of AKI or other kidney diseases.

5.3. Paradigm shift of cell death

There has been an interesting finding published previously that the application of zVAD, a pan-caspase inhibitor, could shift the paradigm of cell death from apoptosis to necroptosis [43]. Researches have demonstrated that apoptosis and regulated necrosis could crosstalk at various molecular levels and therefore could mutually impact each other in some certain conditions. Therefore, researchers and clinicians should be cautious about the unwanted effect in designing cell death inhibition strategies. On the other side, however, it is also reasonable to consider whether the cell death paradigm shifting is a feasible therapeutic modality in AKI treatment. Theoretically, regulated necrosis, unlike apoptosis, can cause the massive release of DAMPs and are thus more inflammatory. Manipulating the cell death profile in favor of reducing structural and functional loss of individuals may provide an optimized treatment effect. This hypothesis, of course, warrants further investigations in the following studies.

6. Conclusions

Taken together, the programmed forms of cell death in AKI consist of apoptosis as well as regulated necrosis that both serve as crucial contributors in renal injuries. An updated and better understanding of the underlying mechanism of regulated cell death provides potential "checkpoints" for AKI treatment. Therapeutic regimens, targeting the regulated cell death, warrant intensive investigations in the near future.

Acknowledgements

This study was supported by National Natural Science Foundation of China (Grants 81400752; 81770746 to CY).

Conflict of interest

The authors declare no conflict of interest.

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