Necroptosis in Acute Kidney Injury

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Keywords

Regulated necrosis · Chronic kidney disease ·
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Abstract

Background/Aims: Regulated necrosis is an expanding research field with important implications for acute kidney injury (AKI). A focused review of the evolving evidence for necroptosis in AKI, one of several forms of regulated necrosis defines the known and unknown. **Methods:** A literature search was performed in PUBMED and ScienceDirect between January 1957 and April 2018 using the following keywords: "acute kidney injury," "necrosis," "necroptosis," "necroinflammation." **Results:** The necroptosis signaling cascade involves a number of proteins including receptor-interacting protein-1 (RIPK1), RIPK3, and mixed lineage kinase domain-like pseudokinase (MLKL) as well as the MLKL regulator RGMb. The existing experimental evidence in AKI based on mice with genetic deletions of these proteins, more or less specific inhibitory compounds, and diverse experimental AKI models is reviewed. **Conclusion:** There is broad consistency suggesting a role for necroptosis in AKI, but some studies report divergent evidence potentially relating to the specific model used and the time point of analysis. *Mkl*-deficient mice are currently the most specific and reliable experimental tool to study necroptosis in vivo

(in kidney disease). The clinical potential of necroptosis inhibition in AKI is to be evaluated, but conceptual problems in AKI definitions and in complex clinical scenarios remain a concern.

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Introduction

The different forms of regulated necrosis and their unique versus interconnected signaling pathways have become an exploring research domain requiring continuous refinements in nomenclature and definitions. The recent version of the recommendations of the Nomenclature Committee on Cell Death 2018 lists 12 major cell death subroutines with unique signal transduction cascades that ultimately lead to cell demise (Fig. 1) [1]. In the kidney research domain, it seems obvious to study regulated necrosis in forms of kidney disease involving necrotic lesions, that is, the different forms of acute kidney injury (AKI). Glomerular loop necrosis occurs in rapidly progressive glomerulonephritis, for example, in renal vasculitis or severe immune complex glomerulonephritis. Vascular necrosis also occurs in thrombotic microangiopathies, for example, in atypical hemolytic uremic syndrome. Kidney embolism leads to territorial necrosis, that is, kidney infarction. Maybe the most prevalent form of

Fig. 1. Major forms of cell death and their pathway-defining molecules. Below the name of the cell death subroutine, a central and specific element of the involved signaling cascade is named. Experimental evidence on the involvement of a pathway is usually based on specific inhibition, deletion, or overexpression of this protein. MLKL, mixed lineage kinase domain-like pseudokinase; GPX4, glutathion peroxidase 4; PARP1, poly(ADP-ribose) polymerase; NET, neutrophil extracellular trap; ROS, reactive oxygen species; ATG, autophagy-related gene; intr., intrinsic; extr., extrinsic; MPT, mitochondrial permeability transition; CYPD, cyclophilin D.

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kidney necrosis is ischemic and toxic acute tubular necrosis mostly affecting the outer stripe of the medulla. In this review, I focus on the evolving evidence for necroptosis, one of the 12 aforementioned cell death subroutines, in the various experimental models of AKI.

Essential Proteins of the Necroptosis Pathway Contributing to AKI

Necroptosis is defined by a specific signaling cascade with necrosome formation as a central element, its upstream triggers, and its downstream effectors (Fig. 2). The evolving evidence for each of these molecules and their molecular interactions has recently been elegantly described in detail [2]. Functional studies require specific modulation of these targets that can be achieved by either genetic targeting through the creation of mutant mice or by the help of pharmaceuticals with some, but often not entirely certain, target specificity.

Receptor-Interacting Protein-1

RIPK1 is a 671 amino acid serine/threonine protein kinase that has a complex regulatory role in cell survival and death. RIPK1 fuels into survival pathways such as nuclear factor-kappa B, Akt, and Janus kinase. The 112 amino acid domain at the C-terminus of RIPK-1 is needed for the interaction with surface receptors such as tumor necrosis factor receptor (TNFR)1 and Fas; hence, RIPK1 becomes a component of the death receptor signaling complex. RIPK1's kinase domain in the 300 amino acid N-terminus is essential for necroptosis. Necrostatin (Nec)-1 and Nec-1s are allosteric inhibitors of kinase activity and, therefore, inhibit necroptosis in

humans and mice, but also other kinase domain-related functions of RIPK-1, for example, the interaction with TNFR-associated factor-2, an element of TNFR-mediated inflammation. *Ripk1*-deficient mice are lethal so to generate genetic evidence for a functional contribution of RIPK1 to AKI it became necessary to generate mice with a targeted mutation in the kinase domain only, that is, *Ripk1KD* (kinase dead) mice. Such mice were found to be largely protected from lethal ischemia-reperfusion (IR)-induced post-ischemic AKI induced by bilateral renal pedicle clamping for 30 min [3] (Table 1). These findings were consistent with data reported from another group in 2 publications using Nec-1 and an identical experimental set-up [4, 5]. As Nec-1 is commercially available and hence widely accessible, other groups have used it in additional models of experimental AKI. Injecting radiocontrast medium 24 h after IR induced further contrast-induced AKI, a type of injury also reversible with Nec-1 [6]. Two independent groups consistently reported that Nec-1 attenuates tubular cell injury in cisplatin-induced AKI in mice [7, 8]. Another group reported Nec-1 to attenuate rhabdomyolysis-induced AKI triggered by intramuscular injection of glycerol [9]. We found Nec-1 to attenuate acute oxalate nephropathy, a murine model of AKI characterized by diffuse calcium oxalate crystal precipitation in the renal cortex and medulla, tubular cell necrosis, and profound interstitial inflammation [10]. Folic acid exposure is another crystal-related toxic AKI model. Martin-Sanchez et al. [11, 12] demonstrated in 2 publications that Nec-1 does not affect the early phase of folic acid-induced tubular cell injury, a phase that is driven predominately by ferroptosis but rather the subsequent inflammatory AKI phase. Obviously, ferroptosis and necroptosis can act in sequence during certain forms

Fig. 2. Necroptosis signaling cascade. IFNR, interferon receptor; TNFR, tumor necrosis factor receptor; LPS, lipopolysaccharide/bacterial endotoxin; DAMP, damage-associated molecular pattern; TLR, toll-like receptor; dsRNA, double-stranded ribonucleic acid; Nec, necrostatin; TRIF, toll/interleukin-1 receptor domain-

containing adaptor protein inducing interferon beta; P, phosphorylation; RIP, receptor-interacting protein; MLKL, mixed lineage kinase domain-like pseudokinase; RGM, repulsive guidance molecule; ESCRT, endosomal sorting complexes required for transport.

of AKI and ferroptosis inhibition increases the protective effect of Nec-1 [13]. Nec-1 has a short half-life; hence, recently a more stable version, Nec-1s, has been preferred to block the kinase domain of RIPK1 in vivo. Indeed, Nec-1s also attenuates the later phase of folic acid-induced AKI in mice [12]. Other inhibitors of RIPK1 with less certain specificity are the protein kinase inhibitor sorafenib tosylate, the flavonoid-like chemical compound wogonin, an extract of the plant *Scutellaria baicalensis*, and the anti-convulsive drug phenytoin. The anti-cancer drug sorafenib was found to inhibit RIPK1 kinase activity and hence necroptosis at lower concentrations, without affecting nuclear factor-kappa B activa-

tion [14]. Indeed, in vivo sorafenib tosylate inhibited TNF-induced systemic inflammation as well as post-ischemic AKI in mice [14]. Screening FDA-approved drugs also identified also phenytoin as necroptosis inhibitor acting on RIPK1 kinase domain in vitro and in vivo [15]. Accordingly, phenytoin attenuated post-ischemic AKI in mice [15]. Wogonin displayed similar efficacy on RIPK1 modulation in vitro and attenuated cisplatin-induced AKI in mice [16]. These data consistently suggest that the kinase domain of RIPK1 contributes to various forms of AKI, although the cell type-specific contribution and the window-of-opportunity for intervention have not yet been systematically explored.

Table 1. Experimental evidence for a role of necroptosis in AKI models

Target	Modulation	Disease model	Injury	Reference
RIPK1	<i>Ripk1KD</i> Nec-1	Post-ischemic AKI	↓	[3]
		Post-ischemic AKI	↓	[4, 5]
		Post-ischemic AKI + contrast medium	↓	[6]
		Oxalate nephropathy	↓	[10]
		Folic acid-induced AKI	Early ≈	[11, 12]
		Folic acid-induced AKI	Late ↓	[11]
		Cisplatin-induced AKI	↓	[7, 8]
		Rhabdomyolysis-induced AKI	↓	[9]
	Nec-1s Sorafenib Wogonin Phenytoin	Folic acid-induced AKI	Late ↓	[11]
		Post-ischemic AKI	↓	[14]
		Cisplatin-induced AKI	↓	[16]
RIPK3	<i>Ripk3-/-</i>	Post-ischemic AKI	↓	[15]
		Nephrotoxic serum nephritis	≈	[23]
		B6→Balb/c kidney transplantation	↓	[17]
		Post-ischemic AKI	↓	[3, 4]
		Oxalate nephropathy	↓	[10]
		Folic acid-induced AKI	≈	[11, 12]
		Cisplatin-induced AKI	↓	[8]
	Sorafenib	MPO-ANCA-induced AKI	↓	[18]
		Post-ischemic AKI	↓	[14]
MLKL	<i>Mlkl-/-</i>	Post-ischemic AKI	↓	[3, 15]
		Folic acid-induced AKI	↑	[12]
		Cisplatin-induced AKI	↓	[8]
		Oxalate nephropathy	↓	[10]
		MPO-ANCA-induced AKI	↓	[18]
RGMb	<i>Rgmb-/-</i>	Post-ischemic AKI	↑	[25]
		Oxalate nephropathy	↑	[25]

AKI, acute kidney injury; RIPK, receptor-interacting protein; KD, kinase dead; Nec, necrostatin; MPO, myeloperoxidase; ANCA, anti-neutrophil cytoplasmic antibody; MLKL, mixed lineage kinase domain-like pseudokinase; RGM, Repulsive guidance molecules.

Receptor-Interacting Protein-3

RIPK3 is a cytoplasmic protein without a dead domain; hence, it does not directly interact with surface receptors. In humans, its kinase domain has 2 phosphorylation sites (S199, S227); in mice there are 3 (S204, T231, S232). RIPK1 kinase activity is essential for RIPK3 phosphorylation in necroptosis. RIPK1 and RIPK3 auto- and transphosphorylate each other, which promotes the formation of the microfilament-like necrosome complex [2]. As such, RIPK3 is essential for necroptosis but is still also involved in other inflammatory pathways. Hence, genetic tools such as the phenotype of *Ripk3*-deficient mice may not only display a contribution of necroptosis but also additional pathways. Nevertheless, *Ripk3*-deficient mice have been used extensively to discuss the role of necroptosis in AKI (Table 1). For example, Newton et al.

[3] and Linkermann et al. [4] consistently reported that lack of *Ripk3* attenuates IR-induced post-ischemic AKI in mice using comparable experimental set-ups. The role of RIPK3 in IR-induced AKI is further supported by another group using experimental kidney transplantation with *Ripk3*-deficient donor kidneys of a C57BL/6 genetic background transplanted into Balb/c mice [17]. Although alloimmunity heavily contributes to renal outcomes, IR injury certainly remains an additional element of kidney injury in this model as well as in delayed graft function in human kidney transplantation. Models of toxic AKI such as oxalate nephropathy or cisplatin-induced AKI showed consistent results, that is, *Ripk3*-deficient mice were protected when compared to wild type controls [8, 10]. In folic acid-induced AKI, *Ripk3*-deficient mice did not display a phenotype in the early phase but was protective in

the later phase of injury, which implies divergent functions in the early and late phase of injury; however, this remains to be confirmed by other groups [11, 12].

Ripk3-deficient mice have also been used in glomerular AKI models, for example, in myeloperoxidase (MPO) anti-neutrophil cytoplasmic antibody (ANCA)-induced glomerular injury. Transplantation of bone marrow harvested from *Ripk3*-deficient mice into wild type mice protected the recipients from glomerular injury [18]. This implies a role of RIPK3-mediated necroptosis for neutrophil necroptosis and neutrophil extracellular trap-related glomerular injury, a concept supported by effective in vitro experiments by the same group [18]. Indeed, neutrophil necroptosis has also been reported using other triggers, for example, phorbol 12-myristate 13-acetate or crystalline microparticles using neutrophils isolated from *Ripk3*- (and *Mlkl*)-deficient mice [19, 20]. It is likely that neutrophil necroptosis also contributes to other forms of AKI as neutrophils and neutrophil extracellular trap formation has been observed in AKI models involving either glomerular or tubular necrosis [21, 22]. Interestingly, *Ripk3*-deficient mice did not display any phenotype in heterologous nephrotoxic nephritis [23], a model induced by an antiserum raised against glomerular antigens with similarities to anti-GBM disease and crescentic glomerulonephritis. The reasons for this remain uncertain and more direct comparisons between models are needed to sort out these inconsistencies.

Mixed Lineage Kinase Domain-Like Pseudokinase

The *MLKL* gene belongs to the protein kinase superfamily, but the protein lacks several residues required for kinase activity; therefore, MLKL is considered a pseudokinase. The pseudokinase domain of human and mouse MLKL has 2 phosphorylation sites. Phosphorylation of RIPK1 recruits MLKL to the necrosome complex where RIPK3 phosphorylates MLKL, a process inducing homotrimerization and localization to membranes of cell organelles including mitochondria as well as to the plasma membrane. Membrane localization of MLKL promotes pore formation if not prevented by the endosomal sorting complexes required for transport (ESCRT)-III machinery that can eliminate membrane-bound MLKL via plasma membrane bubble disposal to sustain cell survival [24]. MLKL is the true non-redundant executor of necroptosis and no other biological function of MLKL has yet been defined. Therefore, *Mlkl*-deficient mice are currently the ultimate experimental tool to generate undisputable evidence for an involvement of necroptosis in AKI. *Mlkl*-deficient mice display a consistent attenuation in the follow-

ing experimental AKI models: post-ischemic AKI (Table 1) [3, 15], cisplatin-induced AKI [8], oxalate nephropathy [10], and MPO-ANCA-induced glomerular injury [18]. The fact that lack of MLKL had a much lesser effect on survival upon post-ischemic AKI as compared to *Ripk1*KD and *Ripk3*^{-/-} mice suggests that the contribution of necroptosis in post-ischemic AKI may indeed be overestimated by using less specific tools such as *Ripk3*-deficient mice or Nec-1 [3]. However, in oxalate nephropathy, the protective effect observed in *Ripk3*^{-/-} and *Mlkl*^{-/-} mice was consistent and hence the relative contribution of necroptosis to AKI is model-specific [10]. Indeed, *Mlkl*^{-/-} mice rather displayed an aggravated phenotype in early folic acid-induced AKI and although a possible contribution in kidney regeneration was discussed, this remains entirely speculative to date [12]. In another publication of the same group, *Mlkl*^{-/-} mice were protected from folic acid-induced AKI when analyzed at a later time point, which suggests a more complex disease pathophysiology that remains to be further clarified [11].

Repulsive Guidance Molecules-b

RGM proteins associate with cell membranes via a glycosphosphatidylinositol (GPI) anchor and serve as co-receptors for several signaling pathways. RGMb has diverse biological functions that relate to its modulatory effect on IL-6 expression and neurite outgrowth but it was recently described as an inhibitor of MLKL homotrimer binding to the plasma membrane, and hence as another endogenous necroptosis inhibitor [25]. Interestingly, RGMb is highly expressed in tubular epithelial cells. As global *RGMb*-deficient mice die in the early postnatal stage, only heterozygous mice or tubular cell-specific knockout mice are used to study AKI. Employing the latter approach, Liu et al. [25] demonstrated an aggravation of post-ischemic AKI or acute oxalate nephropathy, which confirms RGMb as an endogenous inhibitor of tubular injury (Table 1). Elegant in vitro studies demonstrate that lack of RGMb enhances MLKL deposition in tubular cell membranes and the execution of renal cell death. Further studies with other AKI models are eagerly awaited.

Necroptosis as One Component of Necroinflammation in AKI

Necroptosis is only one subroutine of regulated necrosis and numerous other data exist showing that also other forms of regulated necrosis are involved in AKI [26]. As a broader concept, all forms of regulated necrosis induce

inflammation via the release of alarmins and damage-associated molecular patterns (DAMPs) that activate a wide range of different pattern recognition receptors translating these danger signals into the secretion of proinflammatory cytokines and chemokines [27]. This induction of renal inflammation then further drives cell necrosis either directly via the activation of death receptors such as TNFR1 or indirectly via the recruitment of neutrophils, M1 macrophages, and other proinflammatory leukocytes that contribute to kidney injury [27]. The crescendo of renal cell necrosis, DAMP release, and intrarenal inflammation has been referred to as “necroinflammation” [27]. Renal necroinflammation may or may not remain limited to the kidney. Severe injuries release considerable amounts of DAMPs and necrotic cell debris into the circulation and cause remote organ injury, for example, acute pulmonary distress syndrome, via the cytotoxic and proinflammatory effects of extracellular histones or other mediators [28].

Summary

There is knowledge explosion about the molecular mechanisms of AKI that relate to the evolving experimental tools to study the different forms of regulated necrosis and their impact on the tissue inflammation the

dynamics of the interaction loop between cell necrosis and inflammation. This evolving knowledge raises new hopes on targeted interventions to modulate outcomes of AKI patients. Industry has launched huge investments to develop necrosis inhibitors, but none of these compounds has yet been tested in humans. As cell necrosis is also an important clearance mechanism for cells with considerable DNA damage that otherwise could drive malignancies, long-term studies will also be needed to evaluate tumor rate after necrosis inhibition. Altogether, it remains to be seen whether the current definitions of AKI, the complex clinical settings of comorbidities, and co-medications really offer novel therapeutic opportunities. This, however, remains a hope for researchers, physicians, and patients.

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Disclosure Statement

The author declares no conflicts of interest to disclose.

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