54. Huber TB *et al*. mTOR and rapamycin in the kidney: signaling and therapeutic implications beyond immunosuppression. *Kidney Int* 2011; 79: 502–511.
55. Wei C *et al*. Circulating urokinase receptor as a cause of focal segmental glomerulosclerosis. *Nat Med* 2011; 17: 952–960.
56. Ponticelli C *et al*. Posttransplant recurrence of primary glomerulonephritis. *Clin J Am Soc Nephrol* 2010; 5: 2363–2372.
57. Wei C *et al*. Modification of kidney barrier function by the urokinase receptor. *Nat Med* 2008; 14: 55–63.
58. Smeets B *et al*. Parietal epithelial cells participate in the formation of sclerotic lesions in focal segmental glomerulosclerosis. *J Am Soc Nephrol* 2011; 22: 1262–1274.

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Programmed necrosis in acute kidney injury

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Abstract

Programmed cell death (PCD) had been widely used synonymously to caspase-mediated apoptosis until caspase-independent cell death was described. Identification of necrosis as a regulated process in ischaemic conditions has recently changed our understanding of PCD. At least three pathways of programmed necrosis (PN) have been identified. First, receptor-interacting protein kinase 3 (RIP3)-dependent necroptosis causes organ failure following stroke, myocardial infarction and renal ischaemia/reperfusion injury. Necroptosis can be mediated either by a large intracellular caspase-8-containing signalling complex called the *ripoptosome* or by the RIP1-/RIP3-containing *necroptosome* and is controlled by a caspase-8/FLICE inhibitory protein$_{long}$ heterodimer at least in the latter case. Second, mitochondrial permeability transition mediates apoptotic or necrotic stimuli and depends on the mitochondrial protein cyclophilin D. The third PN pathway involves the poly (ADP-ribose) polymerase-calpain axis that contributes to acute kidney injury (AKI). Preclinical interference with the PN pathways therefore raises expectations for the future treatment of ischaemic conditions. In this brief review, we aim to summarize the clinically relevant PCD pathways and to transfer the basic science data to settings of AKI. We conclude that pathologists were quite right to refer to ischaemic kidney injury as 'acute tubular necrosis'.

Keywords: AKI; necroptosis; programmed cell death; RIP1; RIP3

Introduction

From apoptosis to programmed necrosis

Caspase-dependent apoptosis is referred to as 'extrinsic' if it is triggered by death receptors that are members of the tumour necrosis factor receptor (TNFR) superfamily, such as Fas, TNFR1, TRAILR, FN14 and others [1, 2]. These pathways critically involve caspase-8 in mice and both caspase-8 and caspase-10 in humans. 'Intrinsic' apoptosis is independent of death receptors and requires the release of cytochrome *c* from the mitochondrial intermembrane space. Extrinsic and intrinsic apoptosis signalling pathways are interconnected.

The commonly used term 'programmed cell death' (PCD) had been used synonymously to apoptosis until caspase-independent cell death (CICD) was discovered [3]. Within CICD, programmed necrosis (PN) is currently being intensively investigated and our understanding of PCD in general is increasing at an extraordinary pace. The fact that a necrotic cellular phenotype is the result of a genetically determined programme is widely accepted in the basic science community, but the translation into clinical nephrology has yet to be fully performed. From the clinician's point of view, PN opens the door for future therapeutic interference with these pathways, once they are understood and safe inhibitors are generated.

Classical apoptosis

The apoptotic phenotype is characterized by cellular shrinkage, nuclear condensation and membrane blebbing. The extrinsic and intrinsic signalling pathways are known to mediate this characteristic morphology via the activation of caspases (Figure 1).

The extrinsic or death receptor-mediated pathway results in trimerization of death receptors and intracellular formation of the DISC (see Table 1 for common abbreviations) which allows association of adapter molecules, such as TRADD and FADD, which subsequently recruit pro-caspase-8 and FLIP isoforms through interactions of

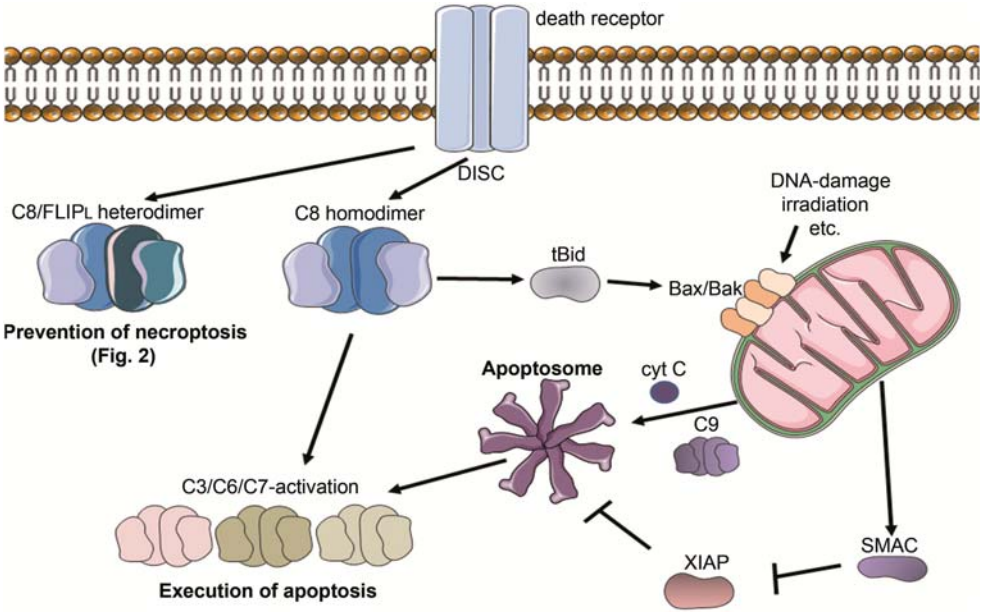

Fig. 1. Model of classical apoptotic signalling. The apoptotic phenotype is characterized by cellular shrinkage, nuclear condensation and membrane blebbing. Two signalling pathways are known to mediate this characteristic morphology via the activation of caspases. The extrinsic or death receptor-mediated pathway results in receptor trimerization and intracellular formation of the DISC that allows association of adapter molecules, which subsequently recruit pro-caspase-8 and isoforms of FLIP. Proximity of two molecules of pro-caspase-8 allows cleavage and activation of via homodimerization of caspase-8 and heterodimerization of caspase-8 with cFLIP. The homodimer cleaves effector caspases-3, -6 and -7 and is responsible for the execution of the apoptotic programme, whereas the heterodimer inactivates CYLD and prevents IAPs from being deubiquitinated, thereby preventing the activation of the necroptotic cascade (caspase-8-non-apoptotic function, see below). The intrinsic apoptotic pathway activates effector caspases by the formation of the *apoptosome*, a complex that consists of caspase-9, Apaf-1 and cytochrome *c*, and is inhibited by XIAP. Whereas caspase-9 becomes activated within this complex, cytochrome *c* and SMAC, an XIAP-inhibitor, are released predominantly from the mitochondrial intermembrane space. This requires the permeability of the outer mitochondrial membrane, e.g. by oligomerization of BH3 proteins like Bax and Bak. Bax/Bak oligomerization follows several stimuli such as DNA damage, irradiation, intracellular calcium overload, glucocorticoid treatment and growth factor depletion. The extrinsic pathway is interconnected with the intrinsic pathway via caspase-8-mediated cleavage of Bid, generating tBID, which is capable of inducing OMM permeabilization.

DD. Induced proximity of two molecules of pro-caspase-8 allows cleavage off the DED of pro-caspase-8 and its activation via homodimerization of caspase-8 and heterodimerization of caspase-8 with cFLIP [4]. The homodimer cleaves and activates effector caspases-3, -6 and -7 and is responsible for the execution of the apoptotic programme, whereas the heterodimer inactivates CYLD and thereby prevents IAPs from being deubiquitinated, thus inhibiting the activation of the necroptotic cascade (caspase-8-non-apoptotic function) [5].

The intrinsic apoptotic pathway activates effector caspases by the formation of the *apoptosome*, an XIAP-controlled supramolecular homodimer composed of two wheel-shaped heptameres that consist of caspase-9, Apaf-1 and cytochrome *c*. Whereas caspase-9 becomes activated within this complex, cytochrome *c* is released predominantly from the mitochondrial intermembrane space and its release requires MOMP, e.g. by oligomerization of BH3 proteins such as Bax and Bak that occurs in response to several stimuli such as DNA damage, irradiation, intracellular calcium overload, activation of Bim, glucocorticoid treatment and growth factor depletion [6]. Upon many of these intrinsic initiators, SMAC/Diablo must required to be activated and inhibit XIAP. Importantly, the extrinsic pathway is interconnected to and amplified by the intrinsic pathway via caspase-8-mediated cleavage of Bid, generating tBID, which is capable of inducing MOMP [6].

Programmed necrosis

Receptor-interacting protein kinase 3-mediated PN (necroptosis)

In apoptotic cells, the caspase-8/FLIP$_{long}$ heterodimer prevents execution of necroptosis by cleavage of CYLD [5] (Figure 2). If caspase-8 is absent or is blocked by viral proteins (e.g. crmA) [7] or synthetic inhibitors (zVAD, qVD or zIETD), CYLD inactivates the E3 polyubiquitin ligases inhibitor of apoptosis protein 1 (IAP1) by deubiquitination. This essentially prevents IAP1/2 from polyubiquitinating RIP1, a critical step in the life/death decision of the cell [8]. Deubiquitinylated RIP1 initiates the assembly of a signalling complex within the cytosol that consists of RIP1 and RIP3 interacting via the RHIM domains in both proteins. In a putative series of phosphorylation events, MLKL is recruited to the complex now referred to as the *necroptosome* [9, 10]. Further, it has been reported that the *necroptosome* activates PGAM5 molecules to transduce the necroptotic signal into mitochondria and induce mitochondrial fragmentation by directly activating Drp-1 [11], but it remains unclear whether this might be a bystander effect and whether mitochondria are critically involved in the execution of necroptosis. However, if mitochondrial fragmentation results in a metabolic and energetic breakdown of the cell, resulting in ATP depletion,

Table 1. List of abbreviations

AIF	Apoptosis-inducing factor
AKI	Acute kidney injury
ANT	Adenine nucleotide translocase
Bnip3L	BCL2/adenovirus E1B 19 kDa protein-interacting protein 3-like
CICD	Caspase-independent cell death
CYLD	Cylindromatosis
DD	Death domain
DED	Death effector domain
DIABLO	Direct IAP-binding protein with low pI
DISC	Death-inducing signalling complex
Drp-1	Dynamin-related protein 1
GFR	Glomerular filtration rate
NEMO	NF-κB essential modulator
NF-κB	Nuclear factor-kappaB
FADD	Fas-associated death domain-containing molecule
FLIP	FLICE inhibitory protein
IAP	Inhibitor of apoptosis protein
IMM	Inner mitochondrial membrane
IRI	Ischaemia-reperfusion injury
LUBAC	Linear ubiquitin chain assembly complex
MEFs	Murine embryonic fibroblasts
MLKL	Mixed lineage kinase domain-like
MMP	Mitochondrial membrane potential
MOMP	Mitochondrial outer membrane permeabilization
MPT	Mitochondrial permeability transition
MPTP	MPT-pore
Nec-1	Necrostatin-1
OMM	Outer mitochondrial membrane
PARP	Poly(ADP-ribose) polymerase
PGAM5	Phosphoglycerate mutase 5
PN	Programmed necrosis
RIP	Receptor-interacting protein kinase
ROS	Reactive oxygen species
RHIM	RIP-homotypic interaction motif
SMAC	Second mitochondria-derived activator of caspases
TWEAK	TNF-like weak inducer of apoptosis
TNF	Tumour necrosis factor
TNFR	Tumour necrosis factor receptor
TRADD	TNFR-associated death domain protein
TRAF2	TNFR-associated factor 2
TRAIL	TNF-related apoptosis-inducing ligand
XIAP	X-linked inhibitor of apoptosis protein

ROS accumulation and a breakdown of the cytosolic electrolyte control, influx of extracellular fluid with consecutive swelling of the cell and its organelles and subsequent plasma membrane rupture might cause the necrotic phenotype. Clearly, rapid and uncontrolled release of the intracellular content into the interstitium causes immunogenic cell death following necroptosis [12].

Two independent groups recently described the receptor-independent assembly of a 2 MDa intracellular platform following application of either synthetic SMAC-mimetics or etoposide which the authors named the *ripoptosome* (Figure 2) [13, 14]. It consists of caspase-8, FADD, the short form of FLIP and RIP1 and is capable of initiating apoptosis (via caspase-8) and necroptosis (via RIP1–RIP3-interaction). Although *in vivo* detection of the *ripoptosome* has not yet been reported, this concept might be applicable to situations in which both apoptosis and necroptosis have been reported to occur in parallel in photoreceptor PCD [15].

Regarding other TNFR superfamily members that are of importance in the kidney, such as Fas or TNF-like weak inducer of apoptosis (TWEAK), at least to our knowledge, no influence of necroptosis has been investigated yet.

Regulation of necroptosis by polyubiquitination

In TNFR-signalling, RIP1 is not only part of PCD but also mediates activation of the NF-kB pathway, a so-called 'survival' pathway. In fact, in over 95% of the settings stimulation of TNFR1 leads to NF-kB activation. But how can a single protein mediate both NF-kB activation which supports cell survival and PCD? The answer is in the ubiquitinylation of RIP1. Polyubiquitinylated RIP1 stabilizes a so-called complex I that involves TRAF2 and activates NEMO (also known as IκB-kinase gamma) to initiate NF-kB signalling. Ubiquitin chains are attached to RIP1 in at least four different settings [16], three of which are of obvious importance to RIP1 signalling. The well-characterized K48 ubiquitinylation mediates proteasomal degradation. K63 ubiquitinylation is mediated by cIAP1/2 under the control of the deubiquitinase CYLD. In addition, linear ubiquitin chains are attached to RIP1 by another complex referred to as LUBAC. It appears that both K63 and linear Ub chains are sufficient to prevent the assembly of the *necroptosome*. Polyubiquitinylation of cell death proteins adds yet another level of complexity to the tight regulation of these pathways [17].

Mitochondria-mediated PN

Calcium and the BH3-only protein Bnip3L-Nix-axis have been demonstrated to trigger necrotic-type cell death. In the case of calcium overload of the cytoplasm, the MPTP opens in a cyclophilin D-dependent manner and leads to sustained release of cytochrome *c* (in contrast to intermittent MPTP opening in intrinsic apoptosis), critical loss of the MMP and activation of a PN phenotype [18]. Intracellular calcium overload has been demonstrated for the ischaemic condition, e.g. *in vivo* in the heart [19, 20]. Because cyclosporine A inhibits cyclophilin D, this pathway has been extensively investigated as a potential therapeutic target in myocardial infarction [21].

The Bnip3L-Nix axis has attracted attention because it is capable of inducing both apoptosis and PN depending on the subcellular distribution. Whereas mitochondrial Nix mediates caspase-dependent apoptosis, the same protein in the endoplasmic reticulum triggered cyclophilin D-dependent PN [22]. Blockade of both caspases and cyclophilin D, therefore, might be an attractive strategy to prevent organ damage in hypoxic settings.

PARP-mediated PN

Apart from the well-described caspase-mediated PARP-1 cleavage in the pathway of classical apoptosis, DNA-alkylating agents trigger the release of AIF from mitochondria, a process that depends on the activation of PARP-1 and active calpains [23] in the absence of active caspases. Intense activation of the DNA repair enzyme PARP-1 leads to PCD by necrosis, possibly mediated by depletion of intracellular ATP [24, 25]. The concise mechanism by which the necrotic programme is transduced is not clear yet. It has been suggested that the PARP-1-mediated PCD

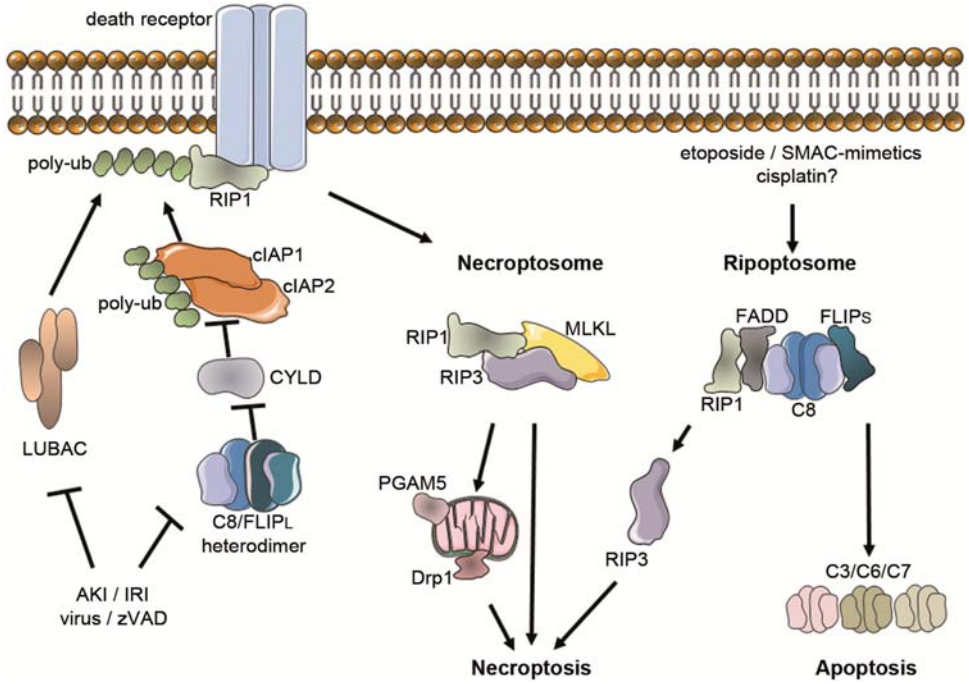

Fig. 2. Model of the signalling pathways of RIP3-dependent necroptosis. In unstimulated cells, death receptor ligation activates the caspase-8/cFLIP heterodimer and prevents execution of necroptosis by inactivation of the deubiquitinase CYLD that allows cIAP1/2 to K63-polyubiquitinylate RIP1. In parallel, LUBAC couples linear ub-chains to RIP1. In the case of viral or pharmacological inhibition of caspase-8 or putatively in the case of AKI or reperfusion after ischaemia, LUBAC might become inactivated and CYLD activity inactivates cIAP1/2. This essentially prevents IAP1/2 from polyubiquitinating RIP1, a process that allows the RHIM-domain of RIP1 to associate with the RHIM-domain of RIP3. Presumably as a consequence of a series of phosphorylation events, RIP1 and RIP3 recruit MLKL to form an intracellular complex referred to as the *necroptosome*. Downstream of the *necroptosome*, PGAM5 molecules might transduce the necroptotic signal into mitochondria and induce mitochondrial fragmentation by activating Drp-1 and causing the energetic breakdown of the cell, a necrotic phenotype with plasma membrane rupture and rapid, uncontrolled release of intracellular content resulting in immunogenic cell death. Upon application of etoposide or SMAC-mimetics and independent of death receptor stimulation, a large 2 MDa complex assembles intracellularly that consists at least of RIP1, FADD, FLIP and RIP1. From this complex, both apoptotic and necroptotic signals may be transduced. The precise regulation of both the *ripoptosome* and the *necroptosome* remain elusive.

is a component of RIP-mediated necroptosis [26], but data from RIP3-deficient MEFs question this hypothesis [27]. It should be mentioned that there is one report that interprets PARP-1 to be upstream of RIP1 [28]. Therein, the authors provide data for the interconnectivity of mitochondrial-mediated and PARP-1-mediated PN by protecting wild-type MEFs from PARP-1-mediated cell death through the addition of cyclosporine A. PARP-1/cyclophilin D double-knockout mice will probably provide a tool to answer these open questions. The clinical relevance of PARP-1-mediated necrosis was mainly established in acute kidney injury (AKI) models [see below and (29, 30)].

Evidence for classical apoptosis in AKI

Apoptosis was investigated in a wide range of AKI models including cisplatin-induced AKI, renal ischaemia-reperfusion injury (IRI) in different settings and others. It is generally accepted that the distal portion of the proximal tubule undergoes both apoptosis and necrosis in AKI, whereas the distal tubules are less necrosis-sensitive and are more likely to undergo apoptosis [31, 32]. As explained in detail subsequently, evidence for the functional contribution of apoptosis to AKI is rather limited, but undoubtedly exists. Whereas morphological assessment of cultured cells lacks specificity and mitochondrial Bax accumulation and cytochrome *c* release may not distinguish between PN and apoptosis [33, 34], clear evidence comes from PI-negative annexin V-positive renal proximal tubular cells (RPTCs) that were treated with cisplatin and from strongly increased caspase activity in the same setting [35]. It is worth mentioning that in the latter study some PI-annexin V-double-positive cells are obvious although the exact percentage is not included. Clearly, protein kinase C delta is a major regulator of cisplatin-induced tubular cell apoptosis, significantly contributes to AKI [35] and might contribute to proteinuria [36]. Increased caspase-3 activity of tubular cells following cisplatin treatment has also been demonstrated by other groups [31, 37].

In several cases, apoptosis is mediated via death receptors that are members of the TNFR family. One of the members, the TWEAK receptor FN14, has been extensively studied by the group of Ortiz and others. It is therefore clear that tubular cells undergo apoptosis upon stimulation with TWEAK in the presence of interferon as demonstrated by zVAD-sensitive activation and cleavage of caspase-8 and caspase-3 and cleavage of Bid [38]. However, this model required the addition of TNFα to the setting. In contrast, whether the protection against renal ischaemia/reperfusion that was mediated by an FN14-targeting monoclonal antibody results from prevention of

apoptosis is much less clear [39, 40]. In the latter paper, the only readout for cell death was TdT-mediated dUTP-biotin nick-end labeling (TUNEL) which might also be positive in PN mediated via the TWEAK-FN14 axis in this setting [39].

In addition to these convincing studies, many of the reports that claimed to have investigated apoptosis in renal IRI have used non-specific readout systems such as TUNEL staining, visual evaluation of apoptotic phenotype, cytochrome *c* release, collapse of the MMP and DNA laddering to conclude that AKI is mediated by apoptosis. These examinations need to be re-evaluated in light of PN. Likewise, slight detection of cleaved caspase-3 can probably be shown from lysates of kidneys that were infiltrated by an immune response wherein classical apoptosis certainly occurs. To prove a functional apoptotic component, a broad-spectrum caspase inhibitor (e.g. zVAD-fmk or q-VD) that significantly reduces serum markers and tubular damage scores should be added to an *in vivo* AKI model [33, 34].

Another upcoming idea discusses endothelial cell-released microvesicles that are capable of reprogramming tubular cells in the sense of downregulating caspase expression and caspase activity by an incompletely understood mechanism. Caspase activity might be regulated in this specific setting, but given the plethora of the non-apoptotic functions of caspases, the direct influence of apoptosis of tubular cells and the relative contribution to the pathophysiological course of AKI also remain to be understood completely [41].

Evidence for PN in AKI

Necroptosis in AKI. In a proximal tubular epithelial cell line (TKPTS), TNFα/zVAD/CHX-induced CICD was specified as necroptosis by the Nec-1-mediated prevention from annexin V positivity [42, 43]. The detection of necroptosis as a relevant *in vivo* mechanism [44–46] gave rise to the hypothesis that organ damage in ischaemic events might be mediated via this pathway. Indeed, Nec-1 protects from renal IRI, whereas zVAD does not [42, 43]. Nec-1 has also been demonstrated to protect renal tubular cells from cisplatin-induced CICD [47]. In line with this, first results in RIP3-deficient mice that have not undergone peer review at the time of writing this review are significantly protected from renal IRI and cisplatin-induced AKI (our unpublished data). Consequently, it will be interesting to investigate RIP3-deficient mice in direct comparison with caspase-8/RIP3 double-deficient mice [48, 49] in AKI models. Additional evidence for the involvement of the necroptotic pathway, especially the necroptotic consequences on mitochondria, retrospectively now arises from an elegant study that employed mice pretreated with a Drp-1 inhibitor (mdivi-1) which were protected from renal IRI and RPTC that were protected from Drp-1-dependent mitochondrial fragmentation [50]. The inducer of necroptosis has not been identified in cisplatin or ischaemic AKI. Because necroptosis can be initiated either via death receptors that are members of the TNFR superfamily [23, 51] or independent of receptors through the *ripoptosome* [13, 14], future experiments will determine which of these are responsible for the induction of AKI. As *ripoptosome* formation was achieved in cell lines by the addition of etoposide, one might speculate on a role for the *ripoptosome* in the pathophysiology of cisplatin-induced AKI. As mentioned earlier, stimulation with TWEAK was shown to induce apoptosis upon addition of interferon. However, addition of a caspase inhibitor to this setting converted the PCD phenotype from apoptosis to dramatic CICD with a necrotic phenotype, typical hallmarks of necroptosis [38, 52]. It remains to be determined whether this PCD subroutine can be identified as necroptosis by addition of Nec-1 or knockdown of RIP3.

Additionally, evidence accumulates for the interconnection of pathways of PN because shRNA of Drp-1, considered to be a downstream player in the necroptotic pathway [11], reduced AIF release in rat RPTC after incubation with azide [53].

Mitochondria-mediated PN in AKI. Cyclophilin D in complex with ANT1 was first isolated form the IMM of kidney cells in a small screen in 2001 [54]. Padanilam *et al*. were the first to transfer the basic science data into kidney research by demonstrating the striking protection of cyclophilin D-deficient mice in renal IRI [55] that has been reproduced by others [56]. The time course of energetic tubular breakdown and precise regulation of MPT in freshly isolated renal tubules from cyclophilin D-deficient mice have subsequently been investigated in a model of hypoxia/reoxygenation [56, 57]. The protective effect of cyclophilin D inhibition by cyclosporine A was overcome by accumulation of hypoxia/reoxygenation-triggered accumulation of non-esterified fatty acids [57]. This might explain why cyclophilin D knockout mice are protected from IRI, whereas cyclosporine A treatment *per se* does not protect, but rather serves as yet another model for tubular cell AKI [58]. In regard to the IRI experiments, it was recently shown that cyclophilin D interacts with glycogen synthase kinase 3β to exert protection from diclofenac-induced ROS production and tubular cell necrosis [59]. In addition to PN, the latter paper discusses an apoptotic component, but none of the following has been investigated: detection of active caspase-3, PI-negative annexin V positivity and inhibition of cell death by caspase inhibitors[59].

PARP-1-mediated PN in AKI. PARP-1-deficient mice show significantly less GFR decline compared with matched controls in a model of renal IRI and the percentage of necrotic cells is reduced [60]. Importantly, in that well-conducted study, the number of nuclei that were positive for DNA-strand breaks and for TUNEL staining 24 h after reperfusion was unchanged in PARP knockout mice [60]. This strongly suggests that necrotic cell death (called immunogenic cell death by others [12]) is the primary damage in ischaemic renal failure followed by a second phase that is characterized by an immune response. The same group demonstrated protection of PARP-1 knockout mice from necrotic cell death, but not apoptosis, in a model of ureteral obstruction [29]. In their most recent work, Kim and Padanilam demonstrated that PARP-1 activation is required for cisplatin nephrotoxicity and described strongly reduced PI positivity of primary tubular cell cultures obtained from

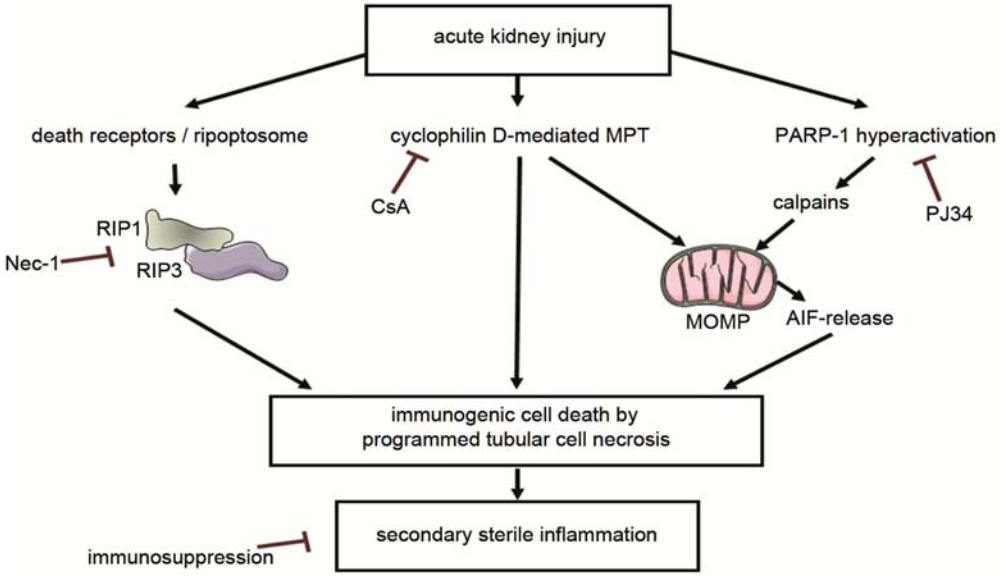

Fig. 3. Programmed necrosis upon renal IRI in the proximal tubular cells. In renal proximal tubular cells, various pathways of PN have been suggested. RIP1-/RIP3-dependent necroptosis kills PTCs in renal IRI and can be interfered with by addition of Nec-1. Constant opening of the MPTP results in cyclophilin D-sensitive programmed necrosis. Cyclosporine A blocks cyclophilin D-mediated MPTP opening. Hyperactive PARP-1 triggers the calpain-AIF axis that also results in CICD and a necrotic phenotype of PTCs in IRI and can be blocked by PARP-1 inhibitors such as PJ34. It is likely that additional blockade of these three pathways yields additional benefits as at least necroptosis and cyclophilin D-mediated MPT are most likely not interconnected, whereas PARP hyperactivation and the mitochondrial pathway show common features.

PARP-1-deficient mice [30]. The authors demonstrated reduction of serum creatinine and urea concentrations *in vivo* in wild-type mice that were treated with the PARP-1 inhibitor PJ34 [61]. It will, therefore, be of interest to see the effect of PARP-1 inhibitors that have been shown to be beneficial in heart transplantation [62], in preclinical IRI models and possibly in clinical trials for the prevention of transplant kidney delayed graft function, but most importantly in combination with Nec-1 and/or cyclosporine A. Drawbacks, however, are ahead, at least in the case of Nec-1, which accelerates time to death in a model of TNFα-mediated shock [63].

Conclusions, open questions and outlook

Regardless of which of the necrotic subroutines described, PN, not apoptosis, appears to be the trigger of immunogenic cell death in various models of AKI (Figure 3). The lesser the primary injury, the lesser the following immune response, causing lesser potentiation of the primary organ failure. Therefore, prevention strategies for AKI should focus on the primary damage and not only on the immune response. Our current concept of the PN in IRI is shown in Figure 3, but many other components that are investigated in basic science, such as ceramide-induced PN, PN caused through the liable iron pool, lysosomal membrane permeabilization, PN caused through ROS and lipid peroxidation, might prove to be of clinical relevance [23, 27].

Basic science data more and more often point out that necroptosis and cyclophilin D-mediated PN are two completely independent pathways that each contribute in their own way to cell damage in combination of the two. Therefore, RIP3/cyclophilin D double-knockout mice and caspase-8/RIP3/cyclophilin D triple-knockout mice will provide powerful tools to investigate the effects of each of these pathway components. Because FADD-, RIP1- and caspase-8-deficient mice are not viable, conditional tubular knockout systems, either inducible or not, are required to unravel the relative contribution of classical apoptosis in AKI.

With respect to PCD in general, interconnections between signalling pathways and cell cycle progression as well as the connection of PCD pathways to autophagy exist, but are beyond the scope of this review. However, the most significant progress in the understanding of PCD pathways will be made by unravelling the complex web of PCD pathways. All transgenic mouse models or applications of drugs *in vivo* that have been used for the investigation of renal IRI are far from providing complete organ protection. Creatinine effects may be misinterpreted because the serum level anticipated by researchers are often 'titrated' to demonstrate optimal effects, e.g. through the time of ischaemia or the dose of cisplatin that is chosen for each experiment. Therefore, a carefully performed pathological evaluation of the tubular damage can be more valuable than creatinine values alone when no glomerular filtration rate is measured. Apart from these general problems, PN in AKI provides an outstanding opportunity to preserve kidney function, especially in conditions where AKI is anticipated, like in renal transplantation, upon application of nephrotoxic drugs or cardiac surgery and before contrast-media-induced AKI.

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References

1. Lamkanfi M, Festjens N, Declercq W *et al*. Caspases in cell survival, proliferation and differentiation. *Cell Death Differ* 2007; 14: 44–55.
2. Boatright KM, Renatus M, Scott FL *et al*. A unified model for apical caspase activation. *Mol Cell* 2003; 11: 529–541.
3. Holler N, Zaru R, Micheau O *et al*. Fas triggers an alternative, caspase-8-independent cell death pathway using the kinase RIP as effector molecule. *Nat Immunol* 2000; 1: 489–495.
4. van Raam BJ, Salvesen GS. Proliferative versus apoptotic functions of caspase-8 Hetero or homo: the caspase-8 dimer controls cell fate. *Biochim Biophys Acta* 2012; 1824: 113–122.
5. Oberst A, Green DR. It cuts both ways: reconciling the dual roles of caspase 8 in cell death and survival. *Nat Rev Mol Cell Biol* 2011; 12: 757–763.
6. Krammer PH, Arnold R, Lavrik IN. Life and death in peripheral T cells. *Nat Rev Immunol* 2007; 7: 532–542.
7. Krautwald S, Ziegler E, Rolver L *et al*. Effective blockage of both the extrinsic and intrinsic pathways of apoptosis in mice by TAT-crmA. *J Biol Chem* 2010; 285: 19997–20005.
8. Challa S, Chan FK. Going up in flames: necrotic cell injury and inflammatory diseases. *Cell Mol Life Sci* 2010; 67: 3241–3253.
9. Sun L, Wang H, Wang Z *et al*. Mixed lineage kinase domain-like protein mediates necrosis signaling downstream of RIP3 kinase. *Cell* 2012; 148: 213–227.
10. Zhao J, Jitkaew S, Cai Z *et al*. Mixed lineage kinase domain-like is a key receptor interacting protein 3 downstream component of TNF-induced necrosis. *Proc Natl Acad Sci USA* 2012; 109: 5322–5327.
11. Wang Z, Jiang H, Chen S *et al*. The mitochondrial phosphatase PGAM5 functions at the convergence point of multiple necrotic death pathways. *Cell* 2012; 148: 228–243.
12. Green DR, Ferguson T, Zitvogel L *et al*. Immunogenic and tolerogenic cell death. *Nat Rev Immunol* 2009; 9: 353–363.
13. Feoktistova M, Geserick P, Kellert B *et al*. cIAPs block *ripoptosome* formation, a RIP1/Caspase-8 containing intracellular cell death complex differentially regulated by cFLIP isoforms. *Mol Cell* 2011; 43: 449–463.
14. Tenev T, Bianchi K, Darding M *et al*. The *ripoptosome*, a signaling platform that assembles in response to genotoxic stress and loss of IAPs. *Mol Cell* 2011; 43: 432–448.
15. Trichonas G, Murakami Y, Thanos A *et al*. Receptor interacting protein kinases mediate retinal detachment-induced photoreceptor necrosis and compensate for inhibition of apoptosis. *Proc Natl Acad Sci USA* 2010; 107: 21695–21700.
16. Gerlach B, Cordier SM, Schmukle AC *et al*. Linear ubiquitination prevents inflammation and regulates immune signalling. *Nature* 2011; 471: 591–596.
17. Emmerich CH, Schmukle AC, Walczak H. The emerging role of linear ubiquitination in cell signaling. *Sci Signal* 2011; 4: re5.
18. Kroemer G, Galluzzi L, Brenner C. Mitochondrial membrane permeabilization in cell death. *Physiol Rev* 2007; 87: 99–163.
19. Baines CP, Kaiser RA, Purcell NH *et al*. Loss of cyclophilin D reveals a critical role for mitochondrial permeability transition in cell death. *Nature* 2005; 434: 658–662.
20. Nakagawa T, Shimizu S, Watanabe T *et al*. Cyclophilin D-dependent mitochondrial permeability transition regulates some necrotic but not apoptotic cell death. *Nature* 2005; 434: 652–658.
21. Piot C, Croisille P, Staat P *et al*. Effect of cyclosporine on reperfusion injury in acute myocardial infarction. *N Engl J Med* 2008; 359: 473–481.
22. Chen Y, Lewis W, Diwan A *et al*. Dual autonomous mitochondrial cell death pathways are activated by Nix/BNip3L and induce cardiomyopathy. *Proc Natl Acad Sci USA* 2010; 107: 9035–9042.
23. Vandenabeele P, Galluzzi L, Vanden Berghe T *et al*. Molecular mechanisms of necroptosis: an ordered cellular explosion. *Nat Rev Mol Cell Biol* 2010; 11: 700–714.
24. Ha HC, Snyder SH. Poly(ADP-ribose) polymerase is a mediator of necrotic cell death by ATP depletion. *Proc Natl Acad Sci USA* 1999; 96: 13978–13982.
25. Moubarak RS, Yuste VJ, Artus C *et al*. Sequential activation of poly(ADP-ribose) polymerase 1, calpains, and Bax is essential in apoptosis-inducing factor-mediated programmed necrosis. *Mol Cell Biol* 2007; 27: 4844–4862.
26. Cabon L, Galan-Malo P, Bouharrour A *et al*. BID regulates AIF-mediated caspase-independent necroptosis by promoting BAX activation. *Cell Death Differ* 2012; 19: 245–256.
27. Vanlangenakker N, Vanden Berghe T, Vandenabeele P. Many stimuli pull the necrotic trigger, an overview. *Cell Death Differ* 2012; 19: 75–86.
28. Xu Y, Huang S, Liu ZG *et al*. Poly(ADP-ribose) polymerase-1 signaling to mitochondria in necrotic cell death requires RIP1/TRAF2-mediated JNK1 activation. *J Biol Chem* 2006; 281: 8788–8795.
29. Kim J, Padanilam BJ. Loss of poly(ADP-ribose) polymerase 1 attenuates renal fibrosis and inflammation during unilateral ureteral obstruction. *Am J Physiol Renal Physiol* 2011; 301: F450–F459.
30. Kim J, Long KE, Tang K *et al*. Poly(ADP-ribose) polymerase 1 activation is required for cisplatin nephrotoxicity. *Kidney Int* 2012; 82: 193–203.
31. Linkermann A, Himmerkus N, Rolver L *et al*. Renal tubular Fas ligand mediates fratricide in cisplatin-induced acute kidney failure. *Kidney Int* 2011; 79: 169–178.
32. Safirstein RL. Am I my brother's keeper?: fratricide in the kidney. *Kidney Int* 2011; 79: 149–150.
33. Galluzzi L, Aaronson SA, Abrams J *et al*. Guidelines for the use and interpretation of assays for monitoring cell death in higher eukaryotes. *Cell Death Differ* 2009; 16: 1093–1107.
34. Galluzzi L, Vitale I, Abrams JM *et al*. Molecular definitions of cell death subroutines: recommendations of the Nomenclature Committee on Cell Death 2012. *Cell Death Differ.* 2012; 19: 107–120.
35. Pabla N, Dong G, Jiang M *et al*. Inhibition of PKCdelta reduces cisplatin-induced nephrotoxicity without blocking chemotherapeutic efficacy in mouse models of cancer. *J Clin Invest* 2011; 121: 2709–2722.
36. Li X, Pabla N, Wei Q *et al*. PKC-delta promotes renal tubular cell apoptosis associated with proteinuria. *J Am Soc Nephrol* 2010; 21: 1115–1124.
37. Kim DH, Jung YJ, Lee JE *et al*. SIRT1 activation by resveratrol ameliorates cisplatin-induced renal injury through deacetylation of p53. *Am J Physiol Renal Physiol* 2011; 301: F427–F435.
38. Justo P, Sanz AB, Sanchez-Nino MD *et al*. Cytokine cooperation in renal tubular cell injury: the role of TWEAK. *Kidney Int* 2006; 70: 1750–1758.
39. Hotta K, Sho M, Yamato I *et al*. Direct targeting of fibroblast growth factor-inducible 14 protein protects against renal ischemia reperfusion injury. *Kidney Int* 2011; 79: 179–188.
40. Weinberg JM. TWEAK-Fn14 as a mediator of acute kidney injury. *Kidney Int* 2011; 79: 151–153.
41. Cantaluppi V, Gatti S, Medica D *et al*. Microvesicles derived from endothelial progenitor cells protect the kidney from ischemia-reperfusion injury by microRNA-dependent reprogramming of resident renal cells. *Kidney Int* 2012; 82: 412–427.
42. Linkermann A, Brasen JH, Himmerkus N *et al*. Rip1 (Receptor-interacting protein kinase 1) mediates necroptosis and contributes to renal ischemia/reperfusion injury. *Kidney Int* 2012; 81: 751–761.
43. Price PM, Hodeify R. A possible mechanism of renal cell death after ischemia/reperfusion. *Kidney Int* 2012; 81: 720–721.
44. Cho YS, Challa S, Moquin D *et al*. Phosphorylation-driven assembly of the RIP1-RIP3 complex regulates programmed necrosis and virus-induced inflammation. *Cell* 2009; 137: 1112–1123.
45. He S, Wang L, Miao L *et al*. Receptor interacting protein kinase-3 determines cellular necrotic response to TNF-alpha. *Cell* 2009; 137: 1100–1111.
46. Zhang DW, Shao J, Lin J *et al*. RIP3, an energy metabolism regulator that switches TNF-induced cell death from apoptosis to necrosis. *Science* 2009; 325: 332–336.
47. Tristao VR, Goncalves PF, Dalboni MA *et al*. Nec-1 protects against nonapoptotic cell death in cisplatin-induced kidney injury. *Ren Fail* 2012; 34: 373–377.

48. Kaiser WJ, Upton JW, Long AB *et al*. RIP3 mediates the embryonic lethality of caspase-8-deficient mice. *Nature* 2011; 471: 368–372.
49. Oberst A, Dillon CP, Weinlich R *et al*. Catalytic activity of the caspase-8-FLIP(L) complex inhibits RIPK3-dependent necrosis. *Nature* 2011; 471: 363–367.
50. Brooks C, Wei Q, Cho SG *et al*. Regulation of mitochondrial dynamics in acute kidney injury in cell culture and rodent models. *J Clin Invest* 2009; 119: 1275–1285.
51. Christofferson DE, Yuan J. Necroptosis as an alternative form of programmed cell death. *Curr Opin Cell Biol* 2010; 22: 263–268.
52. Sanz AB, Sanchez-Nino MD, Ortiz A. TWEAK, a multifunctional cytokine in kidney injury. *Kidney Int* 2011; 80: 708–718.
53. Brooks C, Cho SG, Wang CY *et al*. Fragmented mitochondria are sensitized to Bax insertion and activation during apoptosis. *Am J Physiol Cell Physiol* 2011; 300: C447–C455.
54. Vyssokikh MY, Katz A, Rueck A *et al*. Adenine nucleotide translocator isoforms 1 and 2 are differently distributed in the mitochondrial inner membrane and have distinct affinities to cyclophilin D. *Biochem J* 2001; 358: 349–358.
55. Devalaraja-Narashimha K, Diener AM, Padanilam BJ. Cyclophilin D gene ablation protects mice from ischemic renal injury. *Am J Physiol Renal Physiol* 2009; 297: F749–F759.
56. Park JS, Pasupulati R, Feldkamp T *et al*. Cyclophilin D and the mitochondrial permeability transition in kidney proximal tubules after hypoxic and ischemic injury. *Am J Physiol Renal Physiol* 2011; 301: F134–F150.
57. Feldkamp T, Park JS, Pasupulati R *et al*. Regulation of the mitochondrial permeability transition in kidney proximal tubules and its alteration during hypoxia-reoxygenation. *Am J Physiol Renal Physiol* 2009; 297: F1632–F1646.
58. Neria F, Castilla MA, Sanchez RF *et al*. Inhibition of JAK2 protects renal endothelial and epithelial cells from oxidative stress and cyclosporin A toxicity. *Kidney Int* 2009; 75: 227–234.
59. Bao H, Ge Y, Zhuang S *et al*. Inhibition of glycogen synthase kinase-3beta prevents NSAID-induced acute kidney injury. *Kidney Int* 2012; 81: 662–673.
60. Zheng J, Devalaraja-Narashimha K, Singaravelu K *et al*. Poly(ADP-ribose) polymerase-1 gene ablation protects mice from ischemic renal injury. *Am J Physiol Renal Physiol* 2005; 288: F387–F398.
61. Kim J, Long KE, Tang K *et al*. Poly(ADP-ribose) polymerase 1 activation is required for cisplatin nephrotoxicity. *Kidney Int* 2012; 82: 193–203.
62. Szabo G, Bahrle S, Stumpf N *et al*. Poly(ADP-Ribose) polymerase inhibition reduces reperfusion injury after heart transplantation. *Circ Res* 2002; 90: 100–106.
63. Linkermann A, Brasen JH, De ZF *et al*. Dichotomy between RIP1- and RIP3-mediated necroptosis in tumor necrosis factor-alpha-induced Shock. *Mol Med* 2012; 18: 577–586.

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