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 (FLIP) 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
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 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.

**Programmed necrosis**

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

In apoptotic cells, the caspase-8/FLIPlong 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]. Deubiquitinated 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,
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 2MDa 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 FLIP-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-κB pathway, a so-called ‘survival’ pathway. In fact, in over 95% of the settings stimulation of TNFR1 leads to NF-κB activation. But how can a single protein mediate both NF-κB 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-κB 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 ubiquitinylination mediates proteasomal degradation. K63 ubiquitinylination is mediated by cIAP1/2 under the control of the deubiquitinating enzyme 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 MPT opens in a cyclophilin D-dependent manner and leads to sustained release of cytochrome c (in contrast to intermittent MPT 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 caspses 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 caspasas. 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
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/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 [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-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), TNFa/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 ischemic 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 ischemic 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 ANTI 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-esterfified 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 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 ischemic 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
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. It will, therefore, be of interest to see the effect of PARP-1 inhibitors that have been shown to be beneficial in heart transplantation, 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.

**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.

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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