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Acute kidney injury: a conspiracy of toll-like receptor 4 on endothelia, leukocytes, and tubules

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Abstract

Ischemic acute kidney injury (AKI) contributes to considerable morbidity and mortality in hospitalized patients and can contribute to rejection during kidney transplantation. Maladaptive immune responses can exacerbate injury, and targeting these responses holds promise as therapy for AKI. In the last decade, a number of molecules and receptors were identified in the innate immune response to ischemia-reperfusion injury. This review primarily focuses on one pathway that leads to maladaptive inflammation: toll-like receptor 4 (TLR4) and one of its ligands, high mobility group box protein 1 (HMGB1). The temporal-spatial roles and potential therapeutics targeting this particular receptor-ligand interaction are also explored.

Keywords

Ischemic acute kidney injury; Innate immunity; Damage associated molecular patterns; Toll-like receptor; Transplant rejection

Introduction

Ischemic acute kidney injury (AKI), previously called acute renal failure or acute tubular necrosis, confronts the physician with major clinical challenges and fundamental, unsolved questions. Other than optimizing renal perfusion, there is no therapy for the underlying pathophysiology. Current therapy compensates for the dysfunction of injured kidneys by avoiding fluid overload, correcting acid-base and electrolyte disturbances, and initiating dialysis if necessary. Although there is often some renal recovery, the acute mortality and morbidity due to AKI remains high. Furthermore, studies show that AKI may lead to

progressive chronic kidney disease (CKD) and increased mortality rates in the long term [1]. Recurrent subclinical AKI is also now proposed as a major cause of progressive CKD [2].

A major insight into the pathogenesis of AKI was the recognition that the initial ischemic insult elicits maladaptive responses that exacerbate the injury [3] (Fig. 1). In other words, the ultimate extent of injury is not inevitably determined by the initial insult but also by ensuing maladaptive responses. This simple concept has profound potential clinical implications because, although the initial insult has already occurred when the nephrologist is consulted, the ongoing maladaptive responses may be modified if effective therapy can be developed. These maladaptive responses (Table 1) include inappropriate intrarenal hemodynamics, altered mitochondrial and other metabolic functions [4-6], endothelial dysfunction [7, 8], and tubular obstruction and back-leak [9]. In addition, there is a maladaptive inflammatory response [10-13]. When ischemic AKI occurs during renal transplantation (i.e., during the trauma that caused donor brain death, cold storage of the kidney during transit to the recipient, and warm ischemia during surgical creation of the vascular anastomoses between donor and recipient), the maladaptive inflammatory response may also exacerbate rejection [14-18].

Innate immune response to ischemia

The details of how injury elicits a maladaptive inflammatory response remain a major unsolved question. The role of the innate immune system in the inflammatory response to injury is an active area of research. The innate immune system is the first-line defense against infections and includes a whole host of receptors built to recognize highly conserved motifs of pathogen proteins, lipids, and nucleic acids (called pathogen-associated molecular patterns, or PAMPs). Experimental evidence suggests that injured cells can also alert the innate immune system via several mechanisms in the absence of infection, so-called sterile inflammation. They can change their cell surface so that they activate complement [19, 20], are recognized by natural killer (NK) T cells [21], or express novel antigens that elicit T-cell and/or antibody autoimmunity [22]. Intracellular damage may be detected by intracellular receptors such as Nod1, Nod2, and Nlrp3 [23, 24] and generates reactive oxygen species (ROS) that lead to the activation of proinflammatory genes, such as *TLR4* or *IRF1* [25, 26].

In addition, injured and dying cells release intracellular molecules into the extracellular space where they acquire proinflammatory properties [27] (Fig. 2). These molecules are referred to as damage-associated molecular pattern molecules (DAMPs), or alarmins [25, 28-33]. In addition, extracellular matrix components can also become DAMPs when they are damaged. One example is hyaluronan, which activates proinflammatory receptors when it becomes fragmented during tissue injury [34]. DAMPs and their receptors are promiscuous: one DAMP may be a ligand for several receptors, and one receptor may bind several DAMPs.

The role of DAMPs and their receptors in kidney disease have been reviewed [35]. A number of innate immune system receptors are implicated in the inflammatory response to ischemic injury in the kidney (Table 2). We now discuss in greater detail one pathway that leads to maladaptive inflammation during ischemic AKI. This pathway consists of one particular DAMP, HMGB1, and one of its receptors, TLR4.

TLR4 and HMGB1

The HMGB1-TLR4 interaction is one of the few DAMP-TLR4 interactions documented by biophysical studies [36]. Furthermore, extracellular HMGB1 and TLR4 are proven participants in the pathogenesis of ischemic AKI. HMGB1 expression increases in both murine ischemic AKI [37, 38] and human biopsies taken at implantation of renal transplant

grafts that had suffered ischemic AKI during the transplant process [39]. Furthermore, antibodies against HMGB1 have been shown to ameliorate murine ischemic AKI [37, 40]. Altogether these experiments suggest that during ischemic AKI, HMGB1 is released from its normal intracellular site into the extracellular space where it acquires proinflammatory properties [41].

TLR4, originally discovered as an innate sensor of lipopolysaccharide (LPS), is one of eight known receptors [36] for extracellular HMGB1. Antibodies against TLR4 have been shown to decrease ischemic AKI in mice [42]. In addition, transgenic knockout [38, 39, 43, 44] and two different spontaneous mutations of TLR4 are protective in experimental ischemia-reperfusion injury in mice [42]. The C3H/HeJ and C57BL/10ScNJ strains used in these studies are unrelated by their genealogy [45] and single nucleotide polymorphism (SNP) analysis [46, 47] (Fig. 3). The profound effect of TLR4 mutations in such unrelated mice is a powerful genetic argument for the importance of TLR4 in ischemic AKI. Previous efforts to apply results from a single inbred strain of mice to humans have sometimes been disappointing because of modifier genes [48-50]. Therefore, using mice with such divergent genetic backgrounds makes the effect of modifier genes unlikely.

Furthermore, inactivating human TLR4 mutations in donated kidneys is associated with improved graft function and reduced rejection following renal transplantation [39]. However, the same loss-of-function mutation shown to be associated with a decreased risk of rejection carried an increased risk of severe bacterial infections and opportunistic infections when they are present in the recipient [51]. TLR4 is expressed on at least three different cell types during ischemic AKI: endothelial cells, leukocytes, and renal tubule cells [25, 38, 42, 43, 52]. As discussed below and shown in Fig. 2, TLR4 on each cell type may play a different role in the maladaptive inflammatory response.

TLR4, HMGB1, and endothelia

Within 4 h after ischemia-reperfusion, endothelial TLR4 appears in the outer medulla [25]. This is a critical location during ischemic AKI because it contains the medullary thick ascending limbs and the straight S3 proximal tubules, which are the most vulnerable structures to ischemic injury in both rodents and humans [53-58]. It is also the site of the greatest inflammation [59, 60]. This inflammation requires increased expression of endothelial adhesion molecules, which bind leukocytes in the blood and facilitate their translocation into the renal parenchyma [59]. When antibodies, transgenic knockout, or antisense RNA are used to inactivate particular adhesion molecules, there is less inflammation and decreased functional injury in murine AKI [61-65].

Endothelial TLR4 and extracellular HMGB1 are required for expression of these adhesion molecules. During the first 4 h of reperfusion, adhesion molecules are expressed on the surface of renal endothelia in wild-type mice but not in mice with a deletion mutation of TLR4 [25]. We also found that HMGB1, released by injured renal cells during ischemic AKI, interacts with endothelial TLR4 to activate adhesion molecule expression only by wild-type, but not TLR4-mutant, endothelial cells in vitro and in vivo [25]. Furthermore, conditional knockout of MyD88, a critical intracellular component of TLR4 signaling, on renal endothelia also protects kidneys from ischemic AKI (Chen and Lu, unpublished data). Although our proposed role for endothelial TLR4 has not previously been demonstrated in ischemic AKI, such a role has been shown during sepsis-induced AKI [66], ischemic injury of the cremaster muscle [67], and endotoxin injury of the lung [68].

ROS release is a well-characterized phenomenon in the kidney following ischemia [69-71]. We demonstrated that TLR4 transcription can be induced in cultured endothelial cells exposed to ROS and endoplasmic reticulum stress [25]. Furthermore, the kinetics of

messenger RNA (mRNA) expression in vitro was similar to endothelial TLR4 expression seen in vivo.

Altogether, our observations support the maladaptive role of endothelial activation in ischemic AKI [8, 59, 61, 72-75] and support the following hypothesis: ischemia results in ROS production, which stimulates TLR4 expression on endothelial cells; at 4 h of reperfusion, tubular injury results in the release of HMGB1; HMGB1 interacts with endothelial TLR4 and activates endothelial cells to express adhesion molecules that allow leukocyte infiltration and the maladaptive inflammation that exacerbates ischemic AKI (Fig. 2).

TLR4, HMGB1, and leukocytes

Macrophages have been implicated in the exacerbation of ischemic AKI [10, 59, 61, 74-76] and have been demonstrated to express TLR4 constitutively on their outer membranes [77-80]. We propose that after immigrating via TLR4-activated endothelium, macrophages are stimulated via their TLR4 receptors by ligands released from injured tubules, and thus exacerbate ischemic AKI [42]. This question has been approached using bone marrow chimeras between TLR4 (+/+) and TLR4 (-/-) mice. This technique allows examination of the contribution of TLR4 on radiosensitive bone-marrow-derived cells (presumably leukocytes) independently of the contribution of TLR4 on radioresistant renal parenchymal cells. Using this technique, studies of hepatic ischemic injury revealed requirement for TLR4 (+/+) leukocytes for maximal injury [81]. However, in ischemic AKI, the contribution of TLR4 (+/+) leukocytes is less clear. On the one hand, one laboratory found little effect of TLR4 on leukocytes [38]. However, we [42] and others [43] find a major contribution of radiosensitive leukocytes expressing TLR4.

To resolve this issue, we studied the production of interleukin 6 (IL-6) by renal leukocytes. IL-6 has previously been shown to be a maladaptive cytokine produced by macrophages after ischemic AKI [82, 83]. We developed techniques to isolate leukocytes from murine kidneys with ischemic AKI and found that only TLR4 (+/+) leukocytes produced IL-6. Furthermore, we also demonstrated a role for TLR4 (+/+) macrophages in an in vitro model of ischemic AKI. In this model, proximal tubule cells released HMGB1 after injury by ROS, and this HMGB1 stimulates TLR4 (+/+) but not TLR4 (-/-), macrophages to produce IL-6. This increase in IL-6 from macrophages can be attenuated by glycyrrhizic acid, a direct inhibitor of HMGB1 cytokine activity [84, 85].

The contribution of TLR4 (+/+) leukocytes to the pathogenesis of ischemic AKI, suggested by the above, is consistent with the literature because of the known expression of TLR4 on leukocytes [77-80] and the known contribution of leukocytes to ischemic AKI [10, 59, 61, 74-76]. How leukocytes are activated during ischemic AKI remains to be fully understood. However, our data suggest that one pathway of activation is stimulation of leukocyte TLR4 by HMGB1 released from injured cells.

TLR4, HMGB1, and renal tubules

At 24 h, but not 4 h, following reperfusion, we found TLR4 protein on proximal tubules. To our knowledge, these are the first studies to use double immunofluorescence for TLR4 and markers specific for the proximal tubule (lotus tetragonolobus lectin, or LTA) to localize TLR4. At even later reperfusion times (days 3-5), TLR4 has also been reported on distal tubules and medullary thick ascending limb cells by using anti-Tamm Horsfall antibodies and in situ hybridization for TLR4 [52], and proximal tubules and distal nephron by using anti-TLR4 antibodies and identifying tubules by morphology alone [86]. TLR4 on renal

tubules may allow these cells to produce maladaptive chemokines and cytokines after stimulation by HMGB1 [40].

Although tubular TLR4 would regulate injury after 24 h, it would not affect earlier injury and inflammation. We suggest this earlier injury results from TLR4 expression on renal endothelia, the TLR4-dependent expression of endothelial adhesion molecules, and the ensuing inflammation. The different timing of TLR4 expression by tubules and endothelia reflects the different regulation of this molecule on these two cell types. As mentioned previously, ROS appears to be important for stimulating the early expression of TLR4 on endothelia. On the other hand, TLR4 expression on tubules is stimulated by interferon gamma (IFN- γ) and tumor necrosis factor alpha (TNF- α) [52], which are cytokines expressed late after injury, presumably by infiltrating leukocytes.

Conclusion

Multiple maladaptive responses to ischemia-reperfusion exist in the kidney and exacerbate injury (Table 1). One major source of injury early following reperfusion is the inflammatory response to the injury. The innate immune system recognizes danger signals (DAMPs) via a variety of receptors, leading to the influx of inflammatory cells and release of proinflammatory cytokines. This review focused on the particular roles of TLR4 and HMGB1 in maladaptive inflammation during ischemic AKI (Fig. 2). TLR4 expression is differentially regulated in the different cell types within the kidney (i.e., endothelial, leukocytes, and tubular epithelial cells) and occurs at different time points following reperfusion. Regardless of the cell type involved, TLR4 activation by HMGB1 appears to serve a proinflammatory role in the early stages of ischemia-reperfusion injury.

Understanding these responses may ultimately result in effective therapy for ischemic AKI or prevent delayed graft function and acute rejection following deceased kidney transplant. TLR4 antagonism, primarily with eritoran tetrasodium (E5564), has been demonstrated to decrease excessive inflammatory cytokine release, reduce organ injury, and improve survival in experimental models of sepsis, myocardial ischemia [87], and renal ischemia [88]. However, the role of TLR4 in repair mechanisms is not fully elucidated, and there are reports of impaired healing with TLR4 antagonism in animal models of inflammatory bowel disease [89, 90]. In addition, the role of TLR4 stimulation in modulating autoimmune TH1 responses, allergic TH2 responses, and immunological tolerance of malignancy needs careful consideration when defining the clinical role of TLR4 antagonists. Early clinical trials of E5564 in severe sepsis are promising, but evaluation of its efficacy and safety are ongoing [91]. Finally, inhibitors of HMGB1 release have been shown to improve survival in experimental sepsis (where there is a late accumulation of systemic levels of HMGB1) [92, 93], but they appear to have a more narrow therapeutic window in treating ischemic injury, in which early or pretreatment appears most beneficial [94, 95]. Interestingly, metformin, the well-known diabetes drug that has been demonstrated to have anti-inflammatory effects during toxin-induced AKI, was recently shown to inhibit HMGB1 release in LPS-treated mice and cells [96].

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Fig. 1.

Injury after ischemic acute kidney injury (AKI) is the sum of the initial insult and subsequent maladaptive responses. Future therapies can be directed toward maladaptive responses, including the inflammatory response

Fig. 2.

Toll-like receptor 4-high mobility group box protein 1 (TLR4-HMGB1) in ischemic acute kidney injury (AKI). In response to reactive oxygen species (ROS) released during ischemia/reperfusion, endothelia of the *vasa rectae* express TLR4 within 4 h after reperfusion **(a)**. Renal tubules also express TLR4, but only after 24 h following reperfusion; renal tubular TLR4 expression is a response to interferon gamma (IFN- γ) and tumor necrosis factor alpha (TNF- α) **(b)**. Injury also increases renal tubular production of endogenous TLR4 ligands [or damage-associated molecular pattern molecules (DAMPs)], such as HMGB1 **(c)**, and injured cells release these ligands into the extracellular space **(d)**. These extracellular TLR4 ligands trigger maladaptive responses. They activate TLR4 on endothelial cells **(e)**, which in turn express adhesion molecules **(f)** that facilitate diapedesis of monocytes (macrophages) from blood into the renal interstitial space **(g)**. The endogenous TLR4 ligands (HMGB1) then activate TLR4 on macrophages **(h)** and tubules **(i)**. Activated macrophages and tubules release maladaptive molecules such as interleukin 6 (IL-6) **(j, k)**, which exacerbate injury.

Fig. 3. Mouse family tree. Toll-like receptor 4 (TLR4) deficiency in unrelated C3H/HeOuJ and C57BL/6 J mice results in decreased injury following ischemic acute kidney injury (AKI). As noted on the family tree, these strains are genetically unrelated, adding power to this observation (reprinted from [47], with permission)

Table 1

Maladaptive responses that exacerbate ischemic acute kidney injury (AKI) (see text for references)

Maladaptive renal hemodynamics
Endothelial dysfunction
Maladaptive mitochondrial and other metabolic responses
Production of excessive reactive oxygen species
Tubular obstruction and back-leak
Maladaptive inflammation

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Table 2

Examples of several DAMPs and their proposed receptors in the kidney during ischemic injury (this list is not exhaustive). Modified from [35], with permission

Injury-signal ligand (DAMP)	Proposed receptors
Modification of cell surface:	
Decrease in complement inhibitors	Complement activation [19, 20]
Glycolipids unique to injured cells	NKT cell receptor [76]
Modification of extracellular matrix proteins:	
Fragmented hyaluronan	TLR2, TLR4 [34]
Biglycan	TLR2, TLR4
Tenascin-C	TLR4
Intracellular ligands released by injured cells:	
HMGB1	TLR2, TLR4, TLR9, CD44, RAGE
HSPs (i.e. HSP70, HSP27)	CD14, CD91, TLR2, TLR4, CD40
Uric acid crystals	TLR2, TLR4, CD14
DNA (genomic or mitochondrial)	TLR9
Adenosine, extracellular ATP	P1, P2X, and P2Y receptors
Intracellular signals	
ROS	Nod1, Nod2, NLRP3 [23, 24]
Neutrophil-derived alarmins	
Cathelicidins	TLR7, TLR9, FPR1, FPR2
Defensins	TLR4, CCR6
Lactoferrin	TLR4

DAMP danger associated molecular pattern. *NKT* natural killer T cell, *HMGB1* high mobility group box protein 1, *TLR* toll-like receptor, *RAGE* receptor for advanced glycation end products. *ATP* adenosine triphosphate, *NLRP3* NOD-like receptor family pyrin domain containing 3, *HSPs* heat-shock proteins, *ROS* reactive oxygen species