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See related article, "Growth-Dependent Podocyte Failure Causes Glomerulosclerosis," on pages 1351–1363.

Dying Cells and Extracellular Histones in AKI: Beyond a NET Effect?

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The sequelae of tissue injury caused by hypoxia, ischemia, mechanical stress, or pathogen-induced inflammation are initiated in part by the release of danger/damage-associated molecular patterns (DAMPs) from damaged or dying cells.¹ Typically serving normal functions in cell homeostasis within the cell, these endogenous molecules are recognized as danger signals by cell surface receptors when released into extracellular spaces. In AKI, as well as other kidney disorders and injury to other organs, the ensuing inflammation and recruitment of the immune system contribute to the pathogenesis and resolution of disease. A wide variety of candidate DAMPs and their putative receptors have been identified, and their role in kidney disease is gradually being uncovered¹. While some receptors appear to be selective for DAMPs, others such as toll-like receptors (TLRs)^{2,3} respond to DAMPs and are included among pattern recognition receptors for pathogen-associated molecular patterns (PAMPs). DNA extruded from apoptotic or necrotic cells falls in one of the DAMPs categories, but it also is becoming clear that associated nuclear molecules, such as histones, contribute to tissue injury and disease. In the current issue of *JASN* Allam *et al.*⁴ investigate whether histone release from dying renal cells functions like DAMPs and contributes to AKI in a TLR-dependent manner.

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The appearance of histones in the extracellular space is not well understood but may arise from apoptotic or necrotic cells through passive release (perhaps as dying cells release their cell contents), from proinflammatory cells by active secretion, or as a component of neutrophil extracellular traps (NETs) from infiltrating neutrophils. In a cell death process termed NETosis that seems largely to be involved with clearance of invading pathogens, nuclear DNA and associated proteins (such as histones), some of which are antimicrobial, are extruded from neutrophils into the extracellular space to form fibrous networks, called NETs.⁵ Neutrophils are often the first responders to pathogens and tissue injury and are recruited to inflammatory sites to contain infections by a variety of means, including NETosis.⁶ NETs serve important antibacterial functions, but histones (and perhaps other NET components) may also produce collateral damage to host bystander cells. Although the term NET was originally named for and described in neutrophils, extracellular traps containing DNA and proteins are also released from other cells, including monocytes/macrophages and eosinophils⁷ and may serve as a more generalized defense mechanism. Indeed, extracellular traps and histones, in particular, may play a role in a variety of inflammatory and autoimmune disorders, such as stroke,⁸ systemic lupus erythematosus,⁹ thrombosis,¹⁰ and AKI, although the latter has not yet been examined.

To explore the role of histones in kidney injury, Allam *et al.* conducted a very thorough series of experiments to demonstrate that histones, when released by damaged cells, mediate cell death and inflammation and contribute importantly to postischemic and septic acute kidney.⁴ They first demonstrated that dying tubular epithelial cells release histones and that histones act directly on renal epithelial and endothelial cells grown in culture to induce both apoptotic and necrotic cell death. The pathophysiological relevance of extracellular histones was explored in mice. Direct visualization of local microcirculatory events was established through *in vivo* microscopy of the mouse cremaster muscle in which local application of histones enhanced leukocyte migration and adherence, and immunostaining showed transendothelial migration of neutrophils and monocyte/macrophages. Chemokine-induced chemotaxis and adhesion molecule-induced rolling, adhesion, and transmigration of leukocytes mediate these processes.^{11,12}

Next, Allam *et al.* injected histones directly into the kidney by intra-arterial injection, which led to inflammation, necrosis, and increased proinflammatory cytokine expression in the injected kidney that was prevented by digestion of the histone preparation with activated protein C, a commonly used method for confirming histone-specific effects. The deleterious effects of histones depended on pretreatment of mice with a low dose of LPS to induce TLR2 and TLR4 expression and were absent in TLR2/4 double-null mice, suggesting a role in septic AKI. In an effort to demonstrate cause and effect, the authors injected blocking antibodies and showed that neutralization of extracellular histones, and specifically of

histone H4, prevented tubular injury and loss of renal function in endotoxin-induced AKI and renal ischemia-reperfusion injury (IRI), respectively, thus suggesting a more general role of histones in different forms of AKI.

Importantly, neutralization of histones reduced neutrophil recruitment, which is known to have a pathogenic role in AKI, and the induction of cytokine and chemokine expression in IRI. These *in vivo* studies demonstrate nicely that extracellular histones contribute to the inflammatory process and loss of kidney function in IRI and, in combination with the authors' *in vitro* studies, suggest that released histones act directly on renal epithelial and endothelial cells to induce injury, consistent with similar findings in a lung injury model.¹³

At this point, the more difficult question of whether dying or injured renal cells *in vivo* are the source of extracellular histones remains to be answered. In view of the rapid and extensive infiltration of neutrophils in AKI,¹⁴ it is intriguing to speculate that NETs are a source of extracellular histone accumulation, a concept that has not yet been explored in AKI.

In the next series of elegant experiments, Allam *et al.*⁴ examined signaling mechanisms underlying the direct cellular effects of extracellular histones and showed that histones induce proinflammatory cytokine release through TLR2/4 activation of NF- κ B.¹⁵ To determine which pattern recognition receptors mediate the effect of histones the authors isolated bone marrow-derived dendritic cells (BMDCs) from MyD88 and TRIF (TIR domain-containing adaptor-inducing interferon- β)-deficient mice. MyD88 is the adaptor molecule for all TLRs except TLR3, which signals through TRIF. Histone H4-induced release of TNF- α and IL-6 was completely abrogated in BMDCs from MyD88-deficient but not from TRIF-deficient mice, indicating that TLR3 was not involved. BMDCs from mice deficient in individual genes for TLRs released cytokines in response to H4 stimulation, but BMDCs lacking both TLR2 and TLR4 did not respond to H4 and were completely unable to secrete TNF α and IL-6.

To examine a direct interaction with these receptors, the authors used fluorescently labeled H4 and found that H4 binds independently with high affinity to TLR2 or TLR4. Last, a dual requirement for both TLR2 and TLR4 was demonstrated *in vivo*; injection of H4 failed to increase plasma concentrations of IL-6 and TNF α in TLR2/TLR4 double-null mice. Further investigation will be needed to understand the nature of this apparent receptor cooperativity and the reason why both are needed for a histone-induced proinflammatory state. In addition to the mechanisms that the authors have uncovered, extracellular histones also inhibit efferocytosis,¹⁶ a term coined to describe the clearance of apoptotic cells by phagocytic cells, such as macrophages, which may thereby prolong the acute inflammatory process and tissue injury.

The findings reported in this paper prompt other questions about the mechanisms of kidney injury. For example, what triggers histone release in IRI? If the mechanism bears similarities to NETosis, it is likely that a cell death process cannot go unchecked and requires mediators of regulation whose cell

source and mechanisms of action remain to be identified. In view of the authors' findings on histones and dendritic cells, is histone-induced cytokine release in IRI dependent on the presence of dendritic cells, and do extracellular histones bind to cell surface receptors on other cell types to provoke an inflammatory response?

In summary, these intriguing results demonstrate the important contribution of extracellular histones in directly injuring target cells and causing release of proinflammatory cytokines through TLR2/4. Similar effects of histones have been reported in liver injury,¹⁷ lung injury,¹³ and sepsis.¹⁸ Confirmation that injured or dying kidney epithelial cells release histones *in vivo* requires further study that may also reveal whether release of histones from damaged host cells is comparable to release from dying neutrophils. It is very interesting to consider a role for NETs in mediating tissue injury, as suggested by NET formation in lung injury,¹³ but their role in AKI remains unclear. Neutrophils are key players in kidney IRI; widespread infiltration and activation of neutrophils in the kidney with release of NETs could contribute to or be a primary source of extracellular histones. In addition, the pathophysiological significance of these findings will be reinforced if it is found that the prevailing concentration of extracellular histones in the local microenvironment is sufficient to activate TLR2 and TLR4 following AKI. As such they would offer potential new therapeutic targets for the treatment of various forms of AKI.

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DISCLOSURES

None.

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Phosphate Binders in CKD: Bad News or Good News?

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Hyperphosphatemia has long been considered an important contributor to the mineral and bone disorder associated with CKD (CKD-MBD).¹ Already 50 years ago, Slatopolsky *et al.*² showed that as GFR decreased, fractional phosphate excretion rose because of inhibition of tubular phosphate reabsorption by parathyroid hormone (PTH), thus preventing serum phosphorus to rise. In the last 10 years, fibroblast growth factor 23 (FGF23) has been progressively recognized as another major regulator, if not the most important factor, in controlling renal phosphate excretion and avoiding phosphate retention in CKD.³ FGF23 exerts this action by activating its main receptor, FGFR-1, in the proximal tubular epithelium, with Klotho as an obligatory coreceptor.^{3,4} These adaptive mechanisms allow serum phosphorus to stay normal or near normal until CKD stages 4–5.

The contribution of hyperphosphatemia to the pathogenesis of secondary hyperparathyroidism and hence osteitis fibrosa in CKD is well established. Moreover, hyperphosphatemia favors the development of soft tissue calcifications, including vascular calcification.⁵ Most importantly, a significant association has been identified between high serum phosphorus levels and mortality, both in CKD patients and in the general population.^{5,6} The associations may be both direct and indirect, through concomitant changes in circulating hormone levels such as PTH and FGF23.^{7,8} It must be pointed out, however, there is no high-quality evidence to date based on randomized controlled trials (RCTs) that normalizing serum phosphorus improves hard patient outcomes.⁵ Available evidence is only circumstantial, mostly based on association studies. The recent Kidney Disease—Improving Global Outcomes guideline on CKD-MBD suggests to reduce elevated phosphorus levels toward the normal range in CKD stage 5D and to keep serum phosphorus normal in CKD stages 3–5.⁹ Note, these are suggestions based on weak evidence. Although both extremely high and extremely low serum phosphorus levels clearly are associated with major complications, there is an intermediate gray zone with optimal serum phosphorus targets difficult to define for the different stages of CKD.

Several means are available to reduce high serum phosphorus levels. They include dietary phosphate restriction, use of phosphate binders, and phosphate removal by effective dialysis in patients with ESRD.⁹ In general, limiting oral phosphate intake cannot be achieved effectively without reducing protein intake. This may be useful in CKD stages 3–5, but not necessarily in CKD stage 5D, where it could even do more harm than benefit.¹⁰ In any case, efficient long-term dietary phosphate restriction proves to be difficult in the majority of patients. In those on renal replacement therapy, the use of high-efficiency dialysis procedures alone allows optimal hyperphosphatemia control.¹¹ However, most patients prefer standard dialysis regimens with which phosphate control is generally insufficient.

The prescription of oral phosphate binders therefore remains the principal therapeutic approach.¹² The degree of