Just Look! Intravital Microscopy as the Best Means to Study Kidney Cell Death Dynamics

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Summary: Kidney cell death plays a key role in the progression of life-threatening renal diseases, such as acute kidney injury and chronic kidney disease. Injured and dying epithelial and endothelial cells take part in complex communication with the innate immune system, which drives the progression of cell death and the decrease in renal function. To improve our understanding of kidney cell death dynamics and its impact on renal disease, a study approach is needed that facilitates the visualization of renal function and morphology in real time. Intravital multiphoton microscopy of the kidney has been used for more than a decade and made substantial contributions to our understanding of kidney physiology and pathophysiology. It is a unique tool that relates renal structure and function in a time- and spatial-dependent manner. Basic renal function, such as microvascular blood flow regulation and glomerular filtration, can be determined in real time and homeostatic alterations, which are linked inevitably to cell death and can be depicted down to the subcellular level. This review provides an overview of the available techniques to study kidney dysfunction and inflammation in terms of cell death in vivo, and addresses how this novel approach can be used to improve our understanding of cell death dynamics in renal disease.
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Kidney cell death plays a key role in the pathology of life-threatening renal diseases, such as acute kidney injury (AKI) and chronic kidney disease (CKD). AKI is a major clinical event, characterized by a rapid decrease in glomerular filtration rate (GFR), and associated with the risk of multiple organ failure owing to the accumulation of metabolic waste.[1] The common etiologies of AKI are ischemia-reperfusion injury (IRI), drug-induced renal injury, sepsis, and glomerulonephritis.[2] The prevalence of AKI continuously is increasing,[3] the mortality is high and AKI often leads to long-term complications such as CKD and end-stage renal disease.[4]

In chronic kidney disease of various etiologies, such as glomerulosclerosis, cell death and the decrease in renal function are less rapid. CKD often is characterized by the injury and depletion of glomerular epithelial cells.[5] Podocytes are postmitotic, highly specialized cells that contribute to the integrity of the glomerular filtration barrier.[6] A loss of podocytes leads to the development of proteinuria, which correlates with the decrease in GFR.[7] In addition, proteinuria is associated with tubular[8] and podocyte[9] cell death, owing to albumin overload, which leads to further disease progression. Because podocytes most likely cannot regenerate themselves[10] and their regeneration by renal progenitor cells still is discussed controversially,[11-13] in particular, cell death of glomerular epithelial cells is dramatic in the course of renal disease.

Cell death classically was categorized into two major types: apoptosis and necrosis. Apoptosis is one of the main cell death mechanisms involved in tubular injury[1] and also plays a role in podocyte loss.[14] It is defined as a programed process that involves the activation of several caspase proteases and eventually drives the cell into death.[15] On the contrary, necrosis commonly was classified as an uncontrolled process, leading to cell membrane rupture and triggering an intense immune response.[16] However, intense research over the past decade has shown that necrosis actually may be mediated by several regulated molecular pathways, named necroptosis, ferroptosis, pyroptosis, mitochondria permeability transition, and neutrophil extracellular traps (NET)-induced cell death (NETosis). A detailed report on these new pathways of cell death is beyond the scope of this article but recently was reviewed in detail elsewhere.[1,17-19]

In AKI and CKD a very dynamic interaction between cell death, inflammation, and epithelial and endothelial dysfunction is involved in disease progression.[16,20] To translate basic scientific findings from bench to bedside more efficiently, a novel study approach is needed to capture the dynamic nature of renal pathology and to better understand cell–cell interactions. Therefore, the simultaneous investigation of renal function and morphology in a time-dependent manner is required.

In vivo multiphoton microscopy (MPM) of the living kidney enables simultaneous studies of renal function and morphology, a unique characteristic that is not achieved by any other technique. In vivo imaging of the kidney has

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been used for more than 10 years and has made substantial contributions to our knowledge of kidney physiology and pathophysiology.[21] In contrast to conventional single-photon microscopy, the excitation energy is provided by two or more photons of longer wavelengths, which are absorbed simultaneously. This event requires a high photon density at the focal point, which is achieved by the use of a pulsed laser beam. Several advantages result from this: because light of longer wavelength is less scattered and less powerful, the living tissue is penetrated deeper and is subject to less phototoxicity. In addition, the required photon density for MPM excitation is achievable almost exclusively in the focal point. Therefore, almost no out-of-focus emission is detectable and more sensitive detectors can be used.[22]

Intravital MPM enables the visualization of kidney structures, such as the glomerular and peritubular vasculature, the proximal tubule (PT), the distal tubule, and the collecting duct (CD). In addition, MPM facilitates a high-powered view on the glomerular filtration barrier and the Bowman's capsule, including the precise identification and study of parietal cells, podocytes, mesangial cells, endothelial cells (Fig. 1), and the endothelial glycocalyx.[23-26] Most importantly, it can be used to determine basic renal function and pathology, such as microvascular blood flow,[27] leukocyte rolling and recruitment,[28,29] single-nephron glomerular filtration rate (snGFR),[27] cell death,[30-32] and cell shedding,[24] and the assessment of glomerular[33-37] and peritubular[30] vasculature permeability. Because of the high resolution of this imaging technique, function even can be depicted down to the cell organelle level. MPM has been used successfully to show renin content and release in the living animal,[38] to study endocytosis and transcytosis of albumin in proximal[39] and glomerular epithelial cells,[23] and to visualize mitochondria function including the generation of reactive oxygen species (ROS).[28,40] In addition, MPM recently was used to evaluate intracellular Ca^{2+} changes in vivo using the selective encoding of a green fluorescent protein (GFP)-calmodulin-myosin light chain kinase (M13) fusion protein (GCaMP3), which serves as a calcium indicator in podocytes.[41] Figure 1 shows how intravital MPM can be used to visualize basic renal structures and to determine renal function, such as capillary blood flow.

This review includes the significant contributions of intravital MPM to the investigation of kidney injury, dysfunction, and cell death.

EPITHELIAL DYSFUNCTION

Proximal Tubule Cells in the Center of Necroinflammation

The progression of AKI involves communication between epithelial and endothelial cells with the innate immune system,[42] a process defined as *necroinflammation*.[16] Upon necrotic cell death, the rupture of the cell membrane leads to the release of proinflammatory cytosolic content, so-called danger-associated molecular patterns (DAMPs). DAMPs can be recognized by pattern-recognizing receptors (PRRs), which are expressed on cells of the innate immune system. The stimulation of PRRs leads to enhanced cytokine synthesis and local tissue inflammation.[16] Similar to immune cells, PT cells participate in an injury-induced inflammatory response. Thus, PT cells express PRRs on their surface, such as the toll-like receptor 4 (TLR4), which enable them to sense a proinflammatory environment. Consequently, PT cells are able to respond to endogenous and exogenous stimuli by increasing their ROS and cytokine levels.[42] TLR4 expression mediates IRI in several organs, including the kidney, and TLR4-deficient mice show less leukocyte infiltration, tubular damage, and lower serum creatinine levels in response to ischemic renal injury when compared with wild-type mice.[43] By promoting increased ROS and cytokine levels and the release of DAMPs upon necrotic cell death, PT cells may communicate with other tubular cells downstream of the nephron, with surrounding endothelial cells and with cells of the innate immune system.[16,44]

Intravital MPM recently showed the TLR4-mediated uptake of intravenously injected fluorescent endotoxin into S1 segments of the proximal tubule.[44] Kalakeche et al[44] further investigated the endotoxin-induced oxidative stress within the tubular system. Although S1 proximal tubules were protected from oxidative stress, S2 and S3 segments showed high levels of ROS in response to S1-mediated endotoxin uptake. This suggests a communication between S1 PT cells with downstream portions of the proximal tubule. Furthermore, the TLR1 was expressed exclusively in S2 and S3 segments of the PT, and lipopolysaccharide treatment decreased the TLR1 expression levels in these nephron segments, probably owing to the internalization or the shedding of the receptor. This suggests that S1 proximal tubules are involved mainly in the uptake of endotoxin during sepsis. TLR4-mediated signaling may further generate cytokines, such as tumor necrosis factor α in S1 PT cells to communicate with downstream nephron segments via TLR1 activation.[44]

In an experimental model of pyelonephritis, GFP-expressing *Escherichia coli* bacteria were microinjected into the PT lumen of a superficial nephron.[45] The infection then was monitored using intravital MPM. Adhesion of single *E coli* bacteria on the apical PT wall resulted in a shutdown in blood supply of the adjacent peritubular blood vessels 3 hours after pathogen application. Microdissection followed by pro- and eukaryotic messenger RNA isolation of the affected nephron showed increased cytokine levels. These results suggest a cytokine-mediated communication between proximal tubule and endothelial cells in response to pathogens, which caused direct vasoconstriction in the adjacent capillaries, leaving the affected