Editorial Comments

Whispers and shouts in the pathogenesis of acute renal ischaemia

Michael S. Goligorsky

Departments of Medicine and Pharmacology, Renal Research Institute and Division of Nephrology, New York Medical College, Valhalla, New York, USA

More than half a century of investigations on the pathogenesis of acute renal failure (ARF) have brought about two major concepts, which could be roughly dubbed as vasculocentric and tubulocentric. In the 1950s–1970s the focus of these studies was on the haemodynamic component—vasomotor nephropathy due to the no-reflow, tubuloglomerular feedback and vasoconstriction. In parallel with these studies, but more so in the 1980s–1990s there was a shift of the attention toward the tubular component—necrosis and apoptosis of tubular epithelial cells, tubular obstruction and back-leak. The goal of this review is to unveil the intrinsic coupling between the two components and to develop a unifying view, integrating the contribution of each component to the clinical manifestations of this syndrome. In this vein, the incipient phase of ARF will be putatively linked to the endothelial dysfunction (whispers), whereas the overt oliguric phase of ARF will be largely dependent on the tubular damage (shouts). We shall also attempt to demonstrate that by silencing the phase of ‘whispers’ it may become possible to prevent the ‘shouts’ phase.

Significant tissue injury is caused by oxidative and nitrosative stress

It has been demonstrated that ischaemic and endotoxin-induced renal injury are accompanied by nitrotyrosine formation [1,2]. Apart from inhibiting the activity of several highly susceptible enzymes, i.e. prostacyclin synthase [3], prostaglandin endoperoxide synthase [4] and Mn-SOD [5], detection of nitrotyrosine-modified proteins is indicative of the concomitant oxidative and nitrosative stress resulting in peroxynitrite formation [6]. Noiri et al. [7] have found that peroxynitrite scavenger ebselen reduces (i) nitrotyrosine formation, (ii) DNA damage, (iii) lipid peroxidation and (iv) improves the functional outcome of renal ischaemia, thus lending additional support to the notion that oxidative and nitrosative stress occur in acute renal ischaemia in vivo and are mechanistically involved in the ensuing loss of kidney function. Interestingly, inducible nitric oxide synthase (iNOS) per se can be responsible for generation of Reactive Oxygen Intermediates (ROI), especially, when l-arginine becomes depleted in macrophages [8]. While tubular epithelial cells per se could be the source of nitrosative and oxidative species, it is likely that the bulk of these precursors of peroxynitrite originate from the infiltrating polymorphonuclear cells and macrophages (Figure 1). Homing of these inflammatory cells in the renal parenchyma requires pre-activation of endothelial cells of the local microvasculature. This alone would predict that endothelial activation and expression of addressins should precede any significant tubular injury by the products of oxidative and nitrosative stress.

Several additional lines of evidence indicate the possibility of endothelial dysfunction in ARF. It has been shown that the ischaemic renal vasculature is characterized by a profound loss of the vasorelaxing effect of acetylcholine [9]. Conger et al. have demonstrated that vasorelaxation in response to stimuli generating endothelium-derived relaxing factor was inhibited in ARF [10,11]. In addition, nitric oxide (NO) production in response to bradykinin was found to be suppressed in ischaemic kidneys [1]. Over-expression of ICAM-1 by the vascular endothelium of the ischaemic kidney has been demonstrated and neutralizing anti-ICAM-1 antibodies significantly improved the outcome of renal ischaemia [12]. In addition, we have demonstrated that the endothelium of renal microvessels in ischaemic kidneys showed an enhanced expression of Arginine–Glycine–Aspartic acid (RGD)-binding integrins [13,14]. Collectively, these observations are suggestive of endothelial cell dysfunction occurring in ARF. Its maintenance can be explained by a recent observation made in vitro. Using recombinant endothelial NOS incubated with the
cofactors, calcium and l-arginine (this system generates NO, which is detectable with an NO-sensitive fluorescent indicator, DAF-2), we showed that addition of peroxynitrite to the enzyme results in a dose-dependent inhibition of NO production (Figure 2). This would suggest that the products of oxidative and nitrosative stress in the tubular epithelium may feed back and further aggravate endothelial dysfunction.

Disturbances in renal circulation after renal artery occlusion have been the focus of many investigations. Studies with tracer wash-out techniques produced evidence for preferential reduction in the cortical blood flow in dogs during haemorrhagic hypotension [15]. One hour of renal artery occlusion has been shown to cause a primary disturbance in the post-glomerular perfusion (as studied with fluorescently labelled globulins), thus leading to the damage of tubular epithelia [16]. On the other hand, micropuncture studies of ischaemic kidneys showed a proportional increase in the afferent and efferent arteriolar resistance resulting in the preserved filtration fraction and the fall in glomerular blood flow [17]. Using single-fibre laser Doppler flowmetry, Hellberg et al. demonstrated trapping of Red Blood Cell (RBC) in the outer medulla, causing shunting of blood to the inner medulla [18]. Thus, there is no agreement as to the initiator site in the development of no-reflow in the post-ischaemic renal vasculature. The data presented above, although limited to the superficial cortical vasculature, suggest that functional vasculopathy develops early in the course of reperfusion and occurs simultaneously in different vascular beds, as discussed below.

The earliest video-microscopic study of renal microvascular blood flow after ischaemia was performed by Steinhausen et al. [19]. Analysing RBC velocity in peritubular capillaries 3 days post-ischaemia, these investigators demonstrated that it was decreased to one-third to one-quarter of control values. Unfortunately, no data on the immediate reperfusion period have been published. Using pencil-lens video-microscopy, we continued this line of investigation, focusing on immediate post-occlusion microcirculation in peritubular capillaries—the site responsible for oxygen transport to the proximal tubular epithelium. Minimally invasive intravital video-microscopy produced a set of observations [20] directly demonstrating that (i) erythrocyte velocity in peritubular capillaries exhibited an immediate partial recovery after release of renal artery occlusion, followed by a profound and sustained deceleration of blood flow; (ii) a proportion of capillaries temporarily lost their patency; (iii) motion analysis of blood flow in post-ischaemic capillaries showed shifts from the orthograde to the retrograde directions. Each of these patterns separately and especially their simultaneous co-existence in the post-ischaemic kidney directly explain the mechanics...
of the no-reflow phenomenon (Figure 3). The apparent lack of detectable early vasoconstriction at the level of intralobular arteries suggests that the impediment to peritubular blood flow occurs downstream—at the level of pre-glomerular, glomerular and post-glomerular microvasculature. Indeed, the glomerular microcirculation shows a pattern similar to the peritubular capillary flow: initial recovery of blood flow is followed by its temporal cessation, despite the fact that renal artery occlusion is no longer present. The rate of post-ischaemic recovery of the microcirculation in these capillary beds, however, is different. While glomerular circulation recovers earlier, peritubular capillaries show a significant delay in the recovery of blood flow.

Hence, Leaf’s and Jamison’s ideas from three decades ago [21–23] incriminating damaged endothelium in the development of a ‘no-reflow’ phenomenon at early stages of acute renal ischaemia, seem to be resurrected and have been enriched with a deeper understanding of endothelial cell pathobiology. Using minimally invasive intravital microscopy of glomerular and peritubular capillary blood flow immediately after ischaemic insult to the rat kidney, we documented ‘no-reflow’ phenomenon manifested by the deceleration, cessation or reversal of blood flow, all occurring sporadically in the glomerular and post-glomerular microvasculature. Morphologic analysis revealed the loss of endothelial integrity in the renal microvasculature. It is quite possible that these sites of endothelial denudation are prone to a prolonged vasoconstriction, in a sense acting like resistors in the electrical circuit, thus explaining the sporadic occurrence of cessation and reversal of blood flow. It is not surprising, therefore, that transplantation and engraftment of functionally competent mature endothelial cells into the circulation of post-ischaemic rats (see below), presumably and predominantly at the sites of denudation, resulted in a remarkable protection of the kidney against ischaemic injury [24]. A similar, albeit less profound, effect was achieved by transplantation of surrogate cells expressing a single endothelium-specific enzyme, eNOS. Based on these findings we hypothesized that endothelial dysfunction develops early in the course of ischaemic ARF (Figures 4A and B), manifests structurally in the loss of endothelial integrity and functionally in defective endothelium-dependent vasorelaxation, reminiscent of the phenomenon described by Furchgott and Zawadzki in denuded arteries [25]. Furthermore, the observed renoprotective effect of transplanted endothelial cells leads to the second hypothesis that the pre-existing circulating endothelial cells or endothelial progenitor cells could be ‘boosted’ to improve natural defences against renal ischaemia, thus supplanting the need to transplant exogenous and heterologous cells.

**Regenerative medicine as it may apply to the post-ischaemic kidney**

Therapeutic strategies based on the use of endothelial progenitor cells are rapidly emerging. In rats with acute myocardial ischaemia induced by ligation of the left anterior descending coronary artery,
transplantation of endothelial progenitor cells partially rescued left ventricular function [26]. This effect was attributed to improved angiogenesis in the ischaemic myocardium, although, the possible role of improved vascular function was not addressed in that study.

In diabetic mice, but not in control animals, transplantation of blood-derived angioblasts accelerated the restoration of blood flow to ischaemic hindlimb [27]. This differential response in diabetic and control animals may be related to pre-existing endothelial dysfunction in animals that benefited from angioblast transplantation.

There is ample evidence that circulating stem and haematopoietic stem cells engraft in the kidney, especially under pathological conditions. Recently, two studies utilizing adult bone marrow-derived stem cells used for therapeutic transplantation in the post-ischaemic period have been published [28,29]. Within 24-h post-ischaemia the number of Lin-Sca1+ circulating cells increased >10-fold. Within several days these cells were found to repopulate tubular epithelium in the outer medulla, but not in the cortex. Using ROSA26 mouse and transplanting bone marrow-derived haematopoietic stem cells in the post-ischaemic period, Lin et al. [29] showed the presence of β-galactosidase-positive cells in the regenerating renal tubules 4 weeks after the intervention. Although immediate functional benefits of such a procedure have not been described, it is reasonable to surmise that this therapy may be beneficial in long-term recovery of tubular function. In neither study were transplanted cells detected in the renal microvasculature.

We reasoned that the use of haematopoietic stem cells did not appear to represent the strategy of choice due to the protracted period of differentiation of transplanted cells [30,31] and gave preference to the use of fully differentiated human umbilical vein endothelial cells (HUVEC) for transplantation.

Functional preservation of ischaemic kidney transplanted with mature endothelial cells was remarkable. More recently, we attempted to achieve immediate functional protection using endothelial progenitor cells (M. Arriero-Sanchez, manuscript in preparation). Transplantation of these cells resulted in their microvascular engraftment and amelioration of renal dysfunction. When non-differentiated stem cells were transplanted, no immediate functional protection was observed.

Having proved the main point—the existence of endothelial dysfunction in acute renal ischaemia and the potential for functional salvage by transplanted endothelial cells—it has become much more rational to attempt boosting the mobilization and accelerating the maturation of circulating endothelial progenitor cells.

From this viewpoint, several therapeutics have been shown or can be predicted to protect renal function by increasing the pool of endothelial progenitor cells or their mobilization. Erythropoietin [32], statins [33,34], bone morphogenetic protein [35], Vascular Endothelial Growth Factor (VEGF) [36] and adenosine [37], to name a few. Dr M. Plotkin in our laboratory is studying the role of intrinsic renal stem cells, elusive until recently, in regeneration of the kidney after different insults.

\[Fig. 4.\] Hypothetical summary of the contribution of endothelial dysfunction to the ischaemic damage to the tubular epithelium. (A) Control and ischaemic renal microvasculature stained with silver nitrite. Note the distorted staining pattern in the post-ischaemic vessels. (B) Cartoon depicting the hypothesis on the development of no-reflow and its consequences for the tubular injury.

In vivo injection of HUVEC improves renal function after renal artery cross-clamping

We demonstrated the vulnerability of in vitro, ex vivo and in vivo endothelial cells exposed to pathophysiologically relevant insults, such as oxidative and nitrosative stress or to ischaemia. These stimuli resulted in the loss of integrity of endothelial layers by desquamating or retracting cells. We argued that the loss of integrity
of the endothelial layer, occurring in vivo after acute renal ischaemia, may lead to impaired vasorelaxation and enhanced vasoconstriction, similar to that observed by Furchgott and Zawadzki [25] in vascular segments with denuded endothelium. Given the possibility that the circulating endothelial cells may hom to the sites of endothelial denudation, athymic nude rats received a single injection of HUVEC after the sham-operation or following the release of renal artery clamp. In rats, which received vehicle alone, renal artery cross-clamping resulted in a significant elevation in plasma creatinine concentration (1.36 ± 0.2 vs 0.38 ± 0.05 mg/dl in control) 24 h after clamp release [24]. Intravenous infusion of HUVEC after the release of the renal artery clamp was associated with a significantly lower concentration of plasma creatinine (0.66 ± 0.09 mg/dl). Injection of HUVEC was asso- ciated with the improvement in renal injury, scored in a blinded fashion. These data suggested that circulating exogenous endothelial cells could protect the kidney against ischaemic injury. The mechanism of this protection depended on the implantation of injected endothelial cells in the renal microcirculation. There was a strong correlation between plasma creatinine and the number of implanted endothelial cells (r = 0.85, P = 0.008). These data lend support to the idea of the primacy of endothelial cell dysfunction in acute renal ischaemia (Figure 4).

Collectively, the above experimental data provide a framework for the proposed hypothesis on the key role of endothelial dysfunction in acute renal ischaemia. Uncoupling of eNOS and defective production of NO result in impaired vasorelaxation of renal resistance arteries, diapedesis of polymorphonuclear leukocytes and monocytes, and local procoagulant and proaggregant conditions. Local vasoconstriction is further exaggerated by the loss of endothelial integrity. These vascular events are followed by tubular mechanisms, which include not only the induction of iNOS and increased production of reactive oxygen intermediates in the renal epithelial cells, but also generation of peroxynitrite by infiltrating polymorphonuclear cells and macrophages. Since the vascular events initiate the cascade of reactions, eventually leading to the destruction of tubular epithelial cells, their prevention by transplanting endothelial cells to the renal circulation interrupts the pathophysiologic mechanisms for development of ARF. Hence, these observations strongly suggest that endothelial cell dysfunction is the primary cause of the ‘no-reflow’ phenomenon, which, when ameliorated, results in prevention of tubular epithelial cell injury seen in ARF. The corollary of this finding is that endothelial dysfunction is an instigator of protracted tubular epithelial cell ischaemia and injury.

Although the translation of in vitro and animal experimental data into the language of clinical manifestations of human disease is a precarious process, akin to the proverbial traduttore traditore, the data presented above would suggest that the pathophysiologic underpinning of the incipient and early phases of ARF is the development of endothelial dysfunction (whispers), whereas clinically ‘loud’ later phases are by and large dependent on the tubular dysfunction.

Acknowledgements. Studies in the author’s laboratory were supported in part by NIH grants 45626, 52783, 54602 and 45605 and Westchester Artificial Kidney Foundation. Many important references could not be included due to space limitations.

Conflict of interest statement. None declared.

References

6. Beckman JS, Beckman TW, Chen J et al. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. Proc Natl Acad Sci USA 1990; 87: 1620–1624
Introduction

Knowledge of biofilm formation and its biological role in chronic subclinical inflammation has largely evolved over the past years [1–5]. The development of a biofilm is a very effective way for bacteria to survive facing hostile conditions and to resist biocides and