The endothelial cell in ischemic acute kidney injury: implications for acute and chronic function

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Recent evidence suggests that injury to the renal vasculature may play an important role in the pathogenesis of both early and chronic ischemic acute kidney injury (AKI). Established and new data support the suggestion that vascular injury, in particular, endothelial cell injury, participates in the extent and maintenance of AKI by pathways that are related to vascular tone. Early alterations in peritubular capillary blood flow during reperfusion has been documented and associated with loss of normal endothelial cell function, which can be replaced pharmacologically or with cell replacement interventions. Distorted peritubular capillary morphology is associated with loss of barrier function that may contribute to early alterations in vascular stasis. In addition, ischemia induces alterations in endothelial cells that may promote inflammation and procoagulant activity, thus contributing to vascular congestion. Reductions in microvasculature density may play a critical part in the progression of chronic kidney disease following initial recovery from ischemia/reperfusion-induced AKI. The exact nature of how capillary loss alters renal function and predisposes renal disease is thought to be due at least in part to hypoxia. Finally, the loss of endothelial cell function may represent an important therapeutic target in which nitric oxide, vascular trophic support, and/or endothelial progenitor cells may show potential importance in ameliorating the acute and/or chronic effects of ischemic AKI.

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Acute kidney injury (AKI) has remained a significant health care concern with high mortality rates for several decades. Ischemia due to hypotension or sepsis is the most common cause of human AKI.[1] Despite the central role of tubular injury clearly evident in this syndrome, the hallmark feature of AKI is a reduction in glomerular filtration rate. Therefore, by its very essence, AKI is a phenomenon of altered renal hemodynamics. The renal vasculature following ischemia is influenced by factors deriving from the circulation and/or stressed renal parenchyma. Many studies have focused on the activation of various vasoactive pathways as a consequence of injury.[2,3] There is now an increasingly keen interest in the effect of direct injury to the renal vasculature and its potential role in the pathogenesis of AKI. Therefore, the purpose of this brief review is to highlight recent advances related to renal vasculature injury in the setting of AKI. Emphasis will be placed on alterations in endothelial structure and function and the evidence that these may influence pathogenesis, based primarily on models of ischemia. Further consideration will be given towards a potential link between injured microvasculature and chronic renal function as a basis for the development of chronic kidney disease (CKD).

TUBULAR AND HEMODYNAMIC CONSEQUENCES IN MODELS OF AKI

AKI and recovery has been studied in animal models following ischemia/reperfusion (I/R) brought about by artery clamping or infusion of vasoactive compounds.[4,5] In rats, following renal ischemia, there is an immediate increase in renal vascular resistance and it has been recognized for some time that therapies which reduce renal vascular resistance diminish the extent of injury resulting from I/R.[6–8] Alterations in renal vascular resistance may result from the activity of injured neighboring tubules before the manifestation of tubular death and may be related to altered prostaglandin synthesis, the generation of reactive oxygen species, or activation of inflammatory pathways that are caused by tubular responses to hypoxia. These pathways, and others, have been described previously in recent excellent reviews.[3] The resultant reduction in blood flow exacerbates hypoxia and ultimately contributes to abundant cell death in the outer medullary tubules. Sutton *et al.*[2] have referred to this critical period of altered vascular function as the 'extension phase'

and suggested that this may represent an important therapeutic window in preserving the kidney from prolonged organ failure and/or death.

Vascular damage as a mediator of early or sustained AKI

I/R damages the renal vasculature and compromises renal function; this viewpoint has received increasing attention in the last several years.[2,9–11] Injury to the vasculature can compromise renal function in a number of ways by influencing vascular reactivity, altering capillary barrier function, and modulating inflammatory and coagulation pathways; any or all of these may prolong the underperfused state of the kidney and exacerbate renal injury following I/R.

Studies by Conger *et al.*[5] were among the first to highlight intrinsic alterations in vascular function following I/R injury; these investigators demonstrated that post-ischemic rat kidneys do not autoregulate blood flow and, in fact, manifest vasoconstriction in response to decreased renal perfusion pressure. This occurred even 1 week following recovery from I/R when total renal blood flow had returned to baseline values. Morphological evidence of injury in endothelial cells and smooth muscle cells was present up to 7 days following I/R, induced by renal artery clamping or norepinephrine infusion. The increased constrictor responses could be blocked by Ca^{2+} antagonists.[12] The loss of normal endothelial nitric oxide synthase (NOS) function was demonstrated by a loss of vasodilator responses to acetylcholine and bradykinin.[12] This reduction in normal endothelial function does not appear to be the result of the loss of endothelial NOS (eNOS) protein levels, but may result from inhibition of enzyme activity since bradykinin failed to produce a measurable levels NO in post-ischemic kidneys measured electrochemically. This occurred despite an overall increase in baseline renal NO content that was thought to be the result of inducible NOS (iNOS) activity in the tubular system (for review see Goligorsky *et al.*[13]). Although still unclear, NO production from eNOS may be impaired at the level of enzyme activity or modified by reactive oxygen species to impair normal vasodilatory activity.[14]

The impairment of normal endothelial dependent vasodilator activity has prompted investigators to develop strategies to enhance endothelial function in the setting of AKI. For example, administration of L-arginine, NO-donor molsidomine, or the eNOS cofactor tetrohydrobiopterin can preserve medullary perfusion and attenuate AKI induced by I/R;[15–19] conversely the administration of N^{ω}-nitro-L-arginine methyl ester, an NO blocker, has been reported to aggravate the course of AKI following I/R injury.[20] Although clearly important, these pharmacological studies make it difficult to assess the contribution of eNOS impairment in the overall course of reduced renal function following I/R.

To more thoroughly address the role of endothelial cells following I/R, recent studies have focused on rapid changes in blood flow in the initiation or maintenance of injury in the setting of I/R. Using *in vivo* video microscopy to monitor capillary perfusion, Yamamoto[10] reported that renal perfusion in peritubular vessels was compromised within minutes of reperfusion and is characterized by sluggish and occasionally retrograde blood flow. Using a similar model, Brodsky *et al.*,[9] demonstrated that implantation of endothelial cells to athymic nude rats or other cells which harbor the eNOS gene protected against this early compromise in blood flow, suggesting that endothelial dysfunction contributes to an early compromise in renal hemodynamics.

An intriguing aspect of these studies relates to the possibility that cell-based therapies that replace endothelial cell function may have benefits in AKI. Arriero *et al.*[21] propagated adult stem cells derived from skeletal muscle of Tie-2 GFP mice; *in vitro* cell expansion led to the isolation of an endothelial cell phenotype defined by expression of GFP and other endothelial markers such as Tie-2, flt, flk, and eNOS. Administration of these cells at the time of reperfusion into the aorta through a carotid catheter generated short-term engraftment into the renal vasculature and short-term protection of renal blood flow after I/R injury. It must be pointed out that Togel *et al.*[22] has shown that adult mesenchymal stem cells derived from bone marrow are protective in rats following I/R. However, although many of these cells have endothelial characteristics, they are only transiently found within the kidney and do not appear to engraft. Thus, it is suggested that that the protection offered is humoral. Recently, this group published additional studies suggesting that these types of mesenchymal stem cell may influence renal vascular activity by increasing trophic support for the renal endothelial cells, thereby preserving function.[23]

In addition to alterations in eNOS, at least one other prominent vasoactive factor of endothelial origin has received considerable attention in the setting of I/R. The potent vasoconstrictor endothelin is elevated in peritubular capillary endothelial cells following reperfusion injury and pharmacological inhibition of endothelin significantly reduces functional renal injury in the setting of ischemia reperfusion.[24,25] However, the overall importance is difficult to assess since the protection conferred by antagonists following I/R is generally modest.[26,27]

Altered endothelial function probably also mediates inflammation, a hallmark feature of I/R injury that has been the subject of numerous studies.[2,3,28] Monocyte and macrophage adhesion is evident as early as 2 h post-I/R.[28,29] This adhesion contributes to erythrocyte trapping and hemostasis, prolonging the reduction in renal blood flow and exacerbating tubular injury. I/R injury itself increases the capillary expression of a number of leukocyte adhesion molecules including P- and E-selectin and intercellular adhesion molecule; treatments geared toward reducing endothelial—leukocyte interactions by inhibiting these molecules protect against renal damage in I/R-induced AKI.[29–31]

Renal I/R injury alters cytoskeletal organization of small arterioles and endothelial cells that may relate to the presentation of surface expression molecules.[2,11,32] It has also been suggested that such alterations in cellular morphology disrupt endothelial cell tight junctions as

indicated by the breakdown of VE-cadherin in microvessels of the kidney. *In vivo* two-photon imaging demonstrated a loss of capillary barrier function within 2 h of reperfusion as evidenced by leakiness of high molecular weight dextrans (>300 000 Da) into the interstitial space.[11] Breakdown of barrier function may be the result of matrix metalloproteinase-2 or -9 activation.[33] The potential ramifications of endothelial leakiness in the course of AKI are unclear. Increased edema resulting from leakiness may compromise renal perfusion by compressing peritubular capillaries and/or increasing hemoconcentration and exacerbating erythrocyte trapping.[2]

The formation of microthrombi have been described in renal I/R models as well as in renal transplant biopsies.[2,34] Whether endothelial cell damage promotes thrombus formation in renal I/R remains to be clearly established as there have been few studies that have directly investigated this pathway.

VASCULAR DAMAGE AS A MEDIATOR OF CHRONIC ALTERATIONS IN RENAL FUNCTION FOLLOWING AKI

Following AKI, the complete return of renal function is generally expected if the patient survives the initial insult. However, there a growing appreciation for the chronic effects of AKI in a number of animal models as well as several clinical conditions in which long-term follow-up studies have been carried out.[35] For example, recent data suggest that up to 13 % of patients, following AKI, progress to end-stage renal disease within 3 years; if a pre-existing renal disease is present, progression to end-stage rises to 28% within the same time period (P Eggers, NIDDK, personal communication, 2006). Moreover, pediatric patients following AKI have a high predisposition to progressive renal failure and hypertension,[35,36] while injury in the setting of transplantation (i.e., delayed graft function) represents an independent risk factor for graft survival and the development of post-transplant hypertension.[35,37] These observations suggest that acute injuries to the kidney predispose chronic complications. Work from our laboratory has led to the suggestion that peritubular capillaries are chronically compromised post-ischemia and may contribute to the long-term complications observed following the initial recovery from AKI. Using a microfil technique, we demonstrated that post-ischemic rat kidneys manifested consistent reductions in microvessel density following uncomplicated recovery from I/R injury at 4, 8, and 40 weeks post-surgery.[35,38] In these original studies, vascular dropout by microfil analysis was assessed as reductions in percent surface area on histology sections or with an index comprising intersections of filled structures across arbitrary grid lines; both of these yielded ~30–50% reductions in density.[35] Although there is potential concern with this method, since impaired perfusion could reduce the ability to visualize existing vessels, other approaches utilize immunohistochemistry of endothelial markers to assess vascular density (e.g., CD31/platelet–endothelial cell adhesion molecule, rat endothelial cell antigen) reliably. In the setting of renal I/R, we have observed a comparable percentage in the reduction of vessels utilizing platelet–endothelial cell adhesion molecule staining (D Basile, unpublished data) suggesting good agreement between different methodologies. The reduction in blood-vessel density following I/R is a seemingly simple observation but reveals an important element of the renal response to I/R; although the renal tubular system has impressive capacity for regeneration, the renal vascular system lacks comparable regenerative potential.

FUNCTIONAL CONSEQUENCES OF CAPILLARY LOSS FOLLOWING ACUTE RENAL FAILURE

Among the questions worth considering is the determination of the functional consequences of peritubular capillary dropout following AKI. CKD following ischemic AKI in rats is characterized by the development of interstitial fibrosis and proteinuria that develops with prolonged recovery times following I/R.[35] There is an emerging view that chronic hypoxia, induced by loss of peritubular capillaries or increased vascular resistance, may result in the development of tubulointerstitial fibrosis in renal diseases of diverse etiologies, representing a potential common pathway for disease progression. Hypoxia stimulates several fibrogenic activities including the molecular regulation of fibrogenic factors such as transforming growth factor (TGF)-β and extracellular matrix genes.[39,40] The fibrosis is progressive in nature since the deposition of interstitial scars increases the distance for diffusion to the renal parenchyma and creates more severe hypoxia and fibrosis.[40,41]

Studies from our laboratory have attempted to address the potential role of hypoxia post-I/R. Using the hypoxia-sensitive marker pimondizole, we demonstrated that kidneys of rats following recovery from acute renal failure are more hypoxic, particularly in the outer medulla, than those of corresponding sham-operated control rats.[42] I/R injury carried out with simultaneous unilateral nephrectomy worsened hypoxia following I/R and hastened the development of secondary renal disease. Moreover, treatment of post-ischemic animals with L-arginine, beginning at day 3 following injury, increased renal blood flow, diminished hypoxia, and attenuated the development of secondary renal scarring and proteinuria that occurs with prolonged recovery times.[42]

The feature of peritubular capillary dropout is shared by other models of progressive renal disease leading to interstitial fibrosis such as models induced by angiotensin II infusion, cyclosporine, 5/6 nephrectomy, aging, unilateral ureteral obstruction, and even glomerulonephritis; some of these have also been characterized by increased hypoxia.[39,43–49] In these studies and ours described above, the primary method used to evaluate hypoxia was based on the incorporation of pimonidizole; this compound is reported to form adducts at ppO2 below 10 mm Hg[50] and the resultant adducts identified by immunohistochemical evaluation. Although simple, the method is not ideal since it is

nonquantitative and allows the potential incorporation of histochemical artifacts. Quantitative methodologies such as the use of oxygen electrodes have not been widely applied in these models, perhaps, because comparisons between animals using small probes (and hence limited sampling volume) would be tenuous. Functional-imaging techniques may be helpful to evaluate renal oxygenation quantitatively. For example, blood oxygen level-dependent magnetic resonance imaging utilizes alterations in oxygenated hemoglobin to induce paramagnetic shifts in surrounding protons[51] and may be useful to evaluate renal oxygenation and/or blood flow noninvasively. This approach was recently utilized to evaluate impaired renal oxygenation in patients with chronic renal allograft dysfunction.[52] In summary, although current data using pimonidizole are consistent with increased hypoxia in the setting of vascular dropout, better correlation with quantitative methods, specifically those with clinical utility, should be at the forefront of future investigation.

In addition to hypoxia-induced scarring, several of these models are also characterized by susceptibility to sodium-dependent hypertension. Similarly, we have also demonstrated that rats recovering from acute renal failure are prone to develop sodium-dependent hypertension after their initial recovery, which is associated with a profoundly accelerated secondary CKD (preliminary unpublished data). These observations suggest that recovery from AKI compromises normal sodium excretion. The mechanism of altered sodium handling following initial recovery from AKI is unclear and future studies will be geared toward understanding whether these alterations are due to effects on the renal vasculature. It is possible that these results relate to reductions in renal medullary blood flow that have been associated with sodium-dependent hypertension and are presumed to shift the pressure–natriuresis relationship towards higher arterial blood pressures.[39,53]

WHAT IS THE CAUSE OF CAPILLARY LOSS FOLLOWING ACUTE RENAL FAILURE?

An additional important yet unresolved question relates to the mechanism by which blood-vessel loss occurs in the setting of I/R. Several possibilities exist, including the idea that acute injury results in death of endothelial cells via apoptotic or necrotic mechanisms. Clearly, factors associated with ischemic injury such as TGF-β and tumor necrosis factor-α can stimulate apoptosis in endothelial cells[54,55] and this possibility has been suggested in renal transplant.[56,57] Although these represent probable scenarios, it must be emphasized that as of yet, there have been no published studies demonstrating apoptosis following renal I/R in endothelial cells.

Endothelial cells may also transdifferentiate into fibroblast/myofibroblasts; this process is referred to as endothelial–mesenchymal transition (endo-MT). Evidence for endo-MT is derived from *in vitro* studies in which cultured endothelial cells express markers of myofibroblasts (e.g., smooth muscle actin) or from *in vivo* data in which vascular cells display markers of both endothelial and mesenchymal cells simultaneously. Recent evidence suggests that this process occurs during development and following injury to the vasculature. In mouse kidney, NOS inhibition resulted in peritubular capillaries staining positively for both endothelial markers as well as smooth muscle actin suggesting possible endo-MT.[58–60] Endo-MT has similarities to epithelial–mesenchymal transition, but has received relatively little attention by comparison. Like epithelial–mesenchymal transition, endo-MT is suggested to be stimulated by many factors including TGF-β.[60] Interestingly, immunoneutralization of TGF-β following renal I/R in rats preserved blood-vessel density and also diminished the number of interstitial fibroblasts present in the post-ischemic kidney; this raises the interesting possibility that TGF-β may promote blood-vessel loss by inducing phenotypic transition to a fibroblastic phenotype.[61] Nevertheless, as is the case with cell death, the potential that I/R injury may contribute to endo-MT has not been formally addressed.

Regardless of the mechanism by which endothelial cells are lost, a related and equally interesting question relates to the lack of vascular repair following renal I/R. It is possible that the humoral milieu post-ischemia may either promote vessel loss and/or inhibit repair or regeneration following injury. Consistent with this idea, we have shown evidence in favor of a shift towards the inhibition of angiogenesis, rather than vascular repair following acute injury. Recent studies from our laboratory demonstrated that the classic angiogenic factor, vascular endothelial cell growth factor (VEGF), is inhibited in the post-ischemic kidney for up to 1 week following I/R (D Basile, preliminary unpublished data); this is in contrast to results of other investigators that have shown no effect of I/R on VEGF mRNA at earlier time points and transient (1 day) upregulation of VEGF protein.[62,63] The reduction of VEGF mRNA expression (or lack of induction) following ischemia is intriguing. *In vitro* studies demonstrate that VEGF mRNA expression is stimulated in tubular epithelial cells in response to hypoxia.[64] Despite this, the reduction in VEGF expression following I/R is consistent with several models of CKD associated with vascular rarefaction and persistent hypoxia.[40,45,65,66] In contrast to studies using cultured proximal tubules, Kramer *et al.*[67] exposed rats to hypoxia, which stimulated an increase in the renal expression of erythropoietin mRNA but not VEGF mRNA. Thus, it appears that the canonical signaling pathway by which hypoxia stimulates VEGF *in vitro* is not operational in the kidney *in vivo*. The discrepancy between VEGF responsiveness in cultured kidney cells *versus* the *in vivo* state is not well understood.

In addition to VEGF reduction, we have identified that renal I/R increases the expression of a number of molecules that inhibit VEGF activity including angiostatin[68] and a disintegrin and metalloproteinase with thrombospondin motifs-1 (ADAMTS1) (preliminary data). Finally, and as mentioned above, blockade of TGF-β, which is highly expressed following I/R, tended to protect microvascular

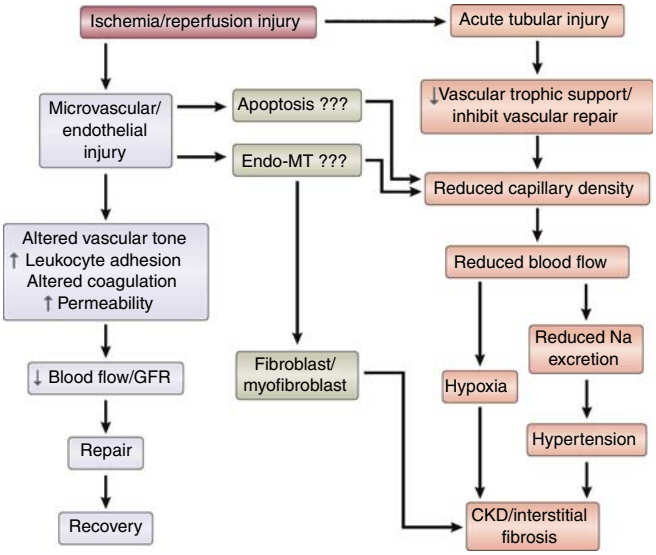

Figure 1 | Putative model for the influence of vascular injury in the setting of renal I/R. The illustration suggests that I/R injury results in damage to both tubular epithelial and vascular cells. Alteration in vascular function results from damage to endothelial and smooth muscle cells that affect early blood flow and contribute to reduced glomerular filtration rate (GFR) and continued injury to the tubular epithelium. Reduction in peritubular capillary density is associated with loss of endothelial cells through undetermined mechanisms. Loss of trophic support or production of inhibitory factors from the epithelium has been proposed. Alteration in capillary structure increases hypoxia-mediated fibrosis and alters proper hemodynamics that contribute to hypertension.

density following I/R.[61] These results suggest that post-ischemic humoral milieu of the kidney may impair regeneration of the vasculature in contrast to the regenerative potential of the tubular system. Current efforts are now geared toward evaluating whether alteration in the post-ischemic milieu can be shifted in favor of vascular repair to preserve or restore capillary density and affect long-term renal function.

Figure 1 illustrates a schematic diagram outlining the potential implications of vascular injury in the setting of I/R. Early alterations in endothelial function may exacerbate the extent of renal injury and further compromise blood flow post-I/R. Resolution of endothelial cells' structure is associated with a resolution in vascular tone and reversal of stasis. Permanent alterations in renal function can be brought about by alterations in endothelial cells that result in loss of capillary numbers. Capillary loss results in alterations in renal oxygenation and hemodynamics that predispose hypertension and CKD. This perspective suggests that interventions targeting endothelial function may be beneficial for both short- and long-term recovery from AKI.

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