Preservation of peritubular capillary endothelial integrity and increasing pericytes may be critical to recovery from postischemic acute kidney injury

Kwon O, Hong S-M, Sutton TA, Temm CJ. Preservation of peritubular capillary endothelial integrity and increasing pericytes may be critical to recovery from postischemic acute kidney injury. Am J Physiol Renal Physiol 295: F351–F359, 2008. First published June 18, 2008; doi:10.1152/ajprenal.90276.2008.—Decreased renal blood flow following an ischemic insult contributes to a reduction in glomerular filtration. However, little is known about the underlying cellular or subcellular mechanisms underlying reduced renal blood flow in human ischemic acute kidney injury (AKI) or acute renal failure (ARF). To examine renal vascular injury following ischemia, intraoperative graft biopsies were performed after reperfusion in 21 cadaveric renal allografts. Confocal fluorescence microscopy was utilized to examine vascular smooth muscle and endothelial cell integrity as well as peritubular interstitial pericytes in the biopsies. The reperfused, transplanted kidneys exhibited postischemic injury to the renal vasculature, as demonstrated by disorganization/disarray of the actin cytoskeleton in vascular smooth muscle cells and disappearance of von Willebrand factor from vascular endothelial cells. Damage to peritubular capillary endothelial cells was more severe in subjects destined to have sustained ARF than in those with rapid recovery of their graft function. In addition, peritubular pericytes/myofibroblasts were more pronounced in recipients destined to recover than those with sustained ARF. Taken together, these data suggest damage to the renal vasculature occurs after ischemia-reperfusion in human kidneys. Preservation of peritubular capillary endothelial integrity and increasing pericytes may be critical to recovery from postischemic AKI.

Subjects

The study group was composed of 21 consecutive consenting recipients of a cadaveric renal allograft who did not subsequently have an episode of acute rejection or other medical or surgical complications. The study was approved by the University of Pittsburgh Institutional Review Boards (IRB 511980) and the Penn State College of Medicine Institutional Review Board (IRB protocol no. 21215). Patients’ ages ranged from 27 to 70 yr. The corresponding age range of the donors varied between 8 and 58 yr.

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Protocol

A wedge biopsy of the allograft was performed approximately 1 h after completion of the vascular anastomosis and restoration of reperfusion of the transplanted kidney in 21 recipients of a cadaveric renal allograft. Graft function was monitored daily following surgery by collecting 24-h urine samples for creatinine clearance (CrCl). Five recipients of a cadaveric renal allograft had severely depressed renal function throughout the first posttransplant week, defined as a CrCl < 25 ml/min (0.42 ml/s) on postoperative day 7. They were designated “sustained AKI.” The other 16 recipients of a cadaveric allograft exhibited recovery of graft function during the first week, characterized by a CrCl ≥ 25 ml/min (0.42 ml/s) on postoperative day 7. This group was designated “recovery.” Renal biopsy specimens from a living related donor of a healthy kidney and two cases undergoing laparoscopic nephrectomy for renal cell cancer with normal kidney function and no systemic or nephrological diseases affecting the kidney were utilized as nonischemic control tissue. Control kidney tissues were obtained before clamping of the renal artery during nephrectomy.

Immunohistochemistry

Antibodies. Texas red-conjugated phalloidin, which stains filamentous actin (F-actin), was purchased from Molecular Probes (Eugene, OR) and used at a dilution of 1:200 to assess the actin cytoskeleton in vascular smooth muscle cells. Monoclonal mouse anti-human von Willebrand Factor (vWF) antibody was purchased from DAKO and used at a dilution of 1:50 to examine the integrity of endothelial cells. Monoclonal mouse anti-α-smooth muscle actin antibody was purchased from Sigma (St. Louis, MO) and used at a dilution of 1:400 to assess peritubular interstitial pericytes or myofibroblasts.

Tissue preparation for immunofluorescence. The fraction of the tissue from the cortex of renal allografts was immediately dropped into 10 ml of 2% paraformaldehyde-0.075 M lysine-0.01 M periodate fixative on ice for 30 min. After fixation, the tissue was washed three times with ice-cold PBS consisting of (in mM) 2.7 KCl, 1.5 KH2PO4, 137 NaCl, and 8 NaH2PO4. Each wash was carried out for 10 min on ice. After this step, the tissue was cryoprotected by transferring it to a 50-ml conical tube containing 40 ml of 2.5 M sucrose in PBS. The tissue was allowed to remain in this solution for at least 48 h at 4°C until the tissue was removed from the 2.5M sucrose solution, immersed in OCT cryoembedding compound (Miles), frozen in liquid N2, and stored at −80°C.

Immunofluorescence staining. The frozen tissue block was mounted on chucks and sectioned using a cryotome (Leica CM 3050, Leica, Nussloch, Germany). Six-micrometer-thick sections were transferred onto ProbeOn Plus glass slides (Fisher Scientific). Frozen sections were then extracted for 10 min at room temperature with a cytoskeleton buffer consisting of (in mM) 50 NaCl, 300 sucrose, 10 PIPES (pH 6.8), 3 MgCl2, and 1 PMSF, as well as 0.5% Triton X-100. The slides were then washed twice with PBS at room temperature. Each wash was carried out for 10 min. After this step, slides were incubated in blocking solution for 2 h at room temperature in a humidified chamber. The blocking solution consisted of PBS containing 20% normal goat serum (NGS), 0.2% BSA, 50 mM NH4Cl, 25 mM glycine, and 25 mM lysine. After the slides were blocked, they were washed twice with PBS containing 0.2% BSA. The washes were carried out for 10 min at room temperature. The slides were then incubated with the appropriate primary antibody solutions overnight at 4°C in a humidified chamber. Primary antibodies were diluted in PBS containing 20% NGS and 0.2% BSA. The following day, slides were again washed twice with PBS containing 0.2% BSA. Both washes were conducted for 10 min at room temperature. The slides were then incubated with the appropriate fluorescein-conjugated secondary antibody and Texas red-conjugated phalloidin for 2 h at room temperature in a humidified chamber for double labeling experiments. Texas red-conjugated phalloidin was used to identify the vascular structure of interest as well as to assess the degree of disorganization of vascular smooth muscle actin. Secondary antibodies were diluted 1:200 in PBS containing 20% NGS and 0.2% BSA. After the secondary antibody incubation, slides were washed twice in PBS containing 0.2% BSA as above and then mounted with glass coverslips in PBS containing 16.7% Mowiol (Calbiochem), 33% glycerol, and 0.1% paraphenylenediamine. Slides were viewed using the MRC-1024 confocal microscope (Bio-Rad, Hercules, CA) with a ×60 water objective lens.

Assessment of Damage to the Renal Vasculature

To evaluate for structural damage to the renal vasculature, serial images were taken at 0.2-μm intervals throughout the 6-μm depth of tissue sections using confocal microscopy. 3D reconstructions of these images were generated with Metamorph software (Universal Imaging, West Chester, PA). Since the degree of damage to the renal vasculature was heterogeneous among cells and among subjects, semiquantitative scoring was utilized as follows. For F-actin staining showing the degree of actin cytoskeletal damage in vascular smooth muscle cells, four blinded observers scored each rotating 3D image using the following criteria: 0, intact filamentous structures in most cells; 1, intact filamentous structure mixed with damaged ones; and 2, damaged structures in most cells. Intact was defined as discernable, distinct filamentous or fibrillar structures and damaged as disorganized, disarrayed, aggregated, clumped, or indiscernible structures. Staining patterns for F-actin showing respective degrees of damage are illustrated (see Fig. 2). For each image, the results of the four blinded observers were averaged. For each case, there were 1–15 images of arteries and 4–22 images of arterioles. Since these images may have had variable scores, each case was then further graded using the following criteria: 0, (mild damage), with scores <1 in ≥70% of the images; 1 (moderate damage), for cases not falling into grades 0 and 2; 2 (severe damage), with scores ≥1 in ≥70% of images. For vWF staining showing the degree of damage to vascular endothelial cells, four blinded observers scored each rotating 3D image using the following criteria: 0, vWF present on >75% of the endothelial lining; 1, vWF present on 50–75% of the endothelial lining; 2, vWF present on 25–49% of the endothelial lining; and 3, vWF present on ≤25% of the endothelial lining. Staining patterns for vWF showing respective degrees of damage are illustrated (see Figs. 3 and 4). By the same reasoning as for actin, each case was then graded by using the following criteria: 0 (mild damage), with scores ≤1 in ≥70% of images; 1 (moderate damage), for cases not falling into grades 0 and 2; 2 (severe damage), with scores ≥2 in ≥70% of images or an average score of the images ≥2.

Assessment of Peritubular Interstitial Pericytes/Myofibroblasts

Semiquantitative scoring of α-smooth muscle actin staining was performed to assess the amount of peritubular pericytes/myofibroblasts. Four blinded observers scored each rotating 3D image using the following criteria: 0, staining for α-smooth muscle actin occupying >75% of the peritubular space; 1, staining for α-smooth muscle actin occupying 50–75% of the peritubular space; 2, staining for α-smooth muscle actin occupying 25–49% of the peritubular space; and 3, staining for α-smooth muscle actin occupying ≤25% of the peritubular space. Staining patterns for pericytes showing respective degrees are illustrated (see Fig. 5). By the same reasoning as for actin and vWF, each case was then graded by using the following criteria: 0 (most prominent staining), with scores ≤1 in ≥70% of images; 1 (moderate staining), for cases not falling into grades 0 and 2; 2 (minimal staining), with scores ≥2 in ≥70% of images or an average score of the images ≥2.
(45/46), and 92.2% (127/138) for arteries, arterioles, and peritubular capillaries, respectively. The agreement rate among four observers within a difference of 2 in scoring of pericyte proliferation was 98.2% (335/341). The agreement rate within a difference of 1 was 68.3%.

**Statistical Analysis**

The statistical significance of difference in physiological or clinical findings between the two different functional groups was tested using an unpaired Student’s t-test. Statistical analyses were performed using SPSS 11.5 software. Results are expressed as means ± SE.

**RESULTS**

**Clinical Features**

Clinical characteristics of the allografts and patient population are summarized in Table 1. Gender and age distribution of patients were similar in subjects displaying either sustained AKI or recovery. Total and warm ischemic times did not differ significantly between sustained AKI and recovery groups (1,693 ± 159 vs. 1,590 ± 77 min and 34 ± 2 vs. 39 ± 2 min, respectively).

**Renal Function**

Subjects were classified into the sustained AKI or recovery groups according to the graft function on postoperative day 7. It was found after analysis that this grouping of subjects already existed on the day of transplantation (Fig. 1). Subjects destined to exhibit sustained AKI displayed a CrCl on day 0 of only 5.6 ± 3.1 ml/min (0.09 ± 0.05 ml/s) compared with a day 0 CrCl of 29.8 ± 3.5 ml/min (0.50 ± 0.06 ml/s) in patients destined to recover rapidly (P = 0.00325). Sustained AKI was also distinguished from recovering function by the observation that the urine flow rate was lower on the day of transplantation in sustained AKI vs. recovery groups (0.8 ± 0.7 vs. 6.9 ± 1.3 ml/min, respectively, P = 0.00027). Among the five patients in the sustained AKI group, four required dialysis during the first posttransplant week. None of the 16 patients in the recovery group required dialysis treatment.

**Cellular Damage to Renal Vasculature**

Our intraoperative biopsy specimen contained tissue samples from superficial and deep cortex of the cadaveric renal allografts. Damage to vascular smooth muscle cells and endothelial cells, as judged by disruption and/or clumping of phalloidin-labeled filaments and absent or dislodged vWF, was observed in all subjects. The degree of damage to vascular smooth muscle and endothelial cells in each functional group varied among individuals and among cells. The grading of the damage to vascular smooth muscle and endothelial cells and pericyte proliferation has been summarized (see Fig. 6). This manuscript also contains supplemental data showing representative rotating 3D images (all supplementary material for this article is available on the journal Web site).

Phalloidin stained many actin filaments in smooth muscle cells and very little in endothelial or epithelial cells except the microvilli of proximal tubule cells.

The degree of damage to renal arteriolar smooth muscle cell actin cytoskeleton. Grade 2 (severe) damage to renal arterial smooth muscle actin was observed in the majority of subjects (11 of 19) (Fig. 2 and see Fig. 6A). No arteries were found in the renal cortical tissue from two subjects. Three of 5 subjects destined to have sustained AKI and 8 of 14 destined to have recovering function showed grade 2 damage. Only one subject in the sustained AKI and two in the recovery group revealed grade 0 (mild) damage. All of the three controls showed grade 0 (mild) damage.

Alteration of renal arteriolar smooth muscle cell actin cytoskeleton. Excluding tissue samples from two subjects in the recovery group that did not contain arterioles, 18 of the 19 samples demonstrated grade 1 (moderate) and 2 (severe) damage to renal arteriolar smooth muscle actin (see Fig. 6B). All five patients destined to have sustained AKI demonstrated grade 2 damage, whereas only 9 of 14 patients destined to have recovery of the graft function showed grade 2 damage and the rest showed grade 1 or grade 0 damage. No patient with sustained AKI demonstrated grade 1 or 0 damage. Two controls showed grade 1 and the other control showed grade 0 damage.

Alteration of renal arterial endothelial cells. The degree of damage to renal arterial endothelial cells varied among individuals in both sustained AKI and recovery groups (Fig. 3 and see Fig. 6C). Fourteen of the 19 cases demonstrated grade 1 (moderate) or 2 (severe) damage. All of the three controls showed grade 0 (mild) damage.

Alteration of renal arteriolar endothelial cells. Injury to renal arteriolar endothelial cells tended to be more severe than that observed in arteries (see Fig. 6D). All subjects examined

![Graph showing Renal Function](http://ajprenal.physiology.org/)

Table 1. Characteristics of patient population

<table>
<thead>
<tr>
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<th>Sustained AKI (n = 5)</th>
<th>Recovery (n = 16)</th>
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<tbody>
<tr>
<td>Women/Men</td>
<td>1/4</td>
<td>4/12</td>
</tr>
<tr>
<td>Age, yr</td>
<td>51 ± 4</td>
<td>47 ± 3</td>
</tr>
<tr>
<td>Total ischemic time, min</td>
<td>1,693 ± 159</td>
<td>1,590 ± 77</td>
</tr>
<tr>
<td>Warm ischemic time, min</td>
<td>34 ± 2</td>
<td>39 ± 2</td>
</tr>
<tr>
<td>Donor age, yr</td>
<td>32 ± 7</td>
<td>32 ± 3</td>
</tr>
</tbody>
</table>

Values are means ± SE. n, No. of subjects; AKI, acute kidney injury.
demonstrated grade 1 (moderate) or 2 (severe) damage. The majority of subjects (3 among 4 subjects in sustained AKI and 11 among 14 in recovery group) demonstrated grade 2 damage to arteriolar endothelial cells. Arteriolar vWF staining could not be identified in one subject with sustained AKI and two subjects with recovering function. Two controls showed grade 0 (mild), and the other control showed grade 1 (moderate) damage.

Alteration of renal peritubular capillary endothelial cells. All five subjects destined to have sustained ARF demonstrated grade 2 (severe) damage to peritubular capillary endothelial cells (Fig. 4 and see Fig. 6E). In contrast, only 9 among 16 subjects with recovering function showed grade 2 damage. One subject with recovering function revealed grade 0 (mild) damage. Two controls showed grade 1, and the other control showed grade 0 damage.
All 7 subjects who had mild to moderate damage to peritubular capillary endothelial cells recovered renal graft function, whereas only 9 among 14 subjects who had the severe damage recovered graft function.

Alteration of Peritubular Pericytes/Myofibroblasts

Peritubular interstitial staining for α-smooth muscle actin, suggesting the presence of pericytes/myofibroblasts, was prominent in subjects with recovering function (Figs. 5 and 6F). Among those with recovering function, 11 of the 16 subjects demonstrated grade 0 (most prominent) staining. In contrast, none of the five destined to have sustained AKI showed grade 0 staining. In subjects with recovering function, grade 1 (moderate) staining was observed in four subjects, and grade 2 (minimal) staining was observed in only one subject. Four of five subjects destined to have sustained AKI showed grade 1 (moderate) staining. The remaining subject in the sustained AKI group demonstrated grade 2 (minimal) staining for pericytes/myofibroblast. All of the three control tissues showed grade 0 (most prominent) staining. All 11 subjects who had the most prominent peritubular interstitial pericyte proliferation recovered renal graft function, whereas only 5 among 10 subjects who had mild to moderate pericyte proliferation recovered graft function. Given that damage to peritubular capillary endothelium and nonproliferation of peritubular pericytes/myofibroblasts were associated with sustained AKI, we examined the combined grades of damage to peritubular capillary endothelial cells (grades 0–2) and degree of pericyte proliferation (grades 0–2) to generate a combined injury score (grades 0–4) for subjects and controls. All 5 subjects destined to have sustained AKI showed a combined injury score of grade 3 or 4, whereas 12 of 16 subjects destined to recover renal graft function showed a combined injury score of grades 0–2. Controls showed a combined injury score of grade 0 or 1.

DISCUSSION

In the current study, ischemic damage to the renal vasculature was assessed by examining alterations in the F-actin of vascular smooth muscle cells and in the distribution of vWF of endothelial cells. In addition, peritubular pericytes or myofibroblasts were examined to assess a response to the ischemic damage. Freshly transplanted cadaveric renal allografts were analyzed since they were known to be the best possible model for postischemic AKI/ATN in humans (1, 11, 19, 20, 38). During the transplant procedure, a previously healthy kidney sustains a period of cold and warm ischemia before implantation. Typically, there are no confounding factors such as sepsis, multiorgan failure, or exposure to nephrotoxins after the initial measurable period of ischemia. As many as 50% of all grafts fail to function promptly following the operation (14). Recipients of a renal allograft in our study were hemodynamically stable without other active medical or surgical conditions which could cause further damage to the kidney within the first 2 post-transplant weeks. Examination of the cellular/subcellular damage to the renal vasculature in AKI has been evasive since gross abnormalities are not usually detected in blood.
vessels of the kidney by light microscopy after an ischemic insult even when physiological abnormalities of renal vasculature, such as increased vascular tone and impaired autoregulatory ability, are prominent. However, myonecrosis of renal arteries and arterioles has been observed at the ultrastructural level in rats with ischemic AKI (9, 23). More recently, renal vascular endothelial injury has been reported in a mouse model of ischemic AKI by an increase in F-actin aggregates and loss of localization in vascular endothelial cadherin immunostaining (36). Data from experimental models of ischemia in animals have demonstrated ultrastructural changes in renal vascular endothelial and smooth muscle cells and aberrant vasomotor tone and reactivity over the first 6 h to 1 wk of reperfusion following ischemia (7–10).

We performed immunohistochemical analysis of biopsy tissues obtained from cadaveric renal allografts following an average of 27 h of ischemia and ~60 min of reperfusion. Examination of these allograft biopsies demonstrated that all the renal vasculature sustained cytoskeletal damage of smooth muscle cells as determined by alterations in F-actin filaments as well as damage to endothelial cells reflected in dislodged or absent vWF. The severity of damage varied among subjects and among cells. However, disrupted, disorganized, aggregated actin filaments were observed in vascular smooth muscle cells of resistance blood vessels, i.e., interlobular arteries and arterioles of all subjects. This finding corresponds to the previous report showing alterations of actin filaments in renal vascular smooth muscle cells after ischemia in rats (21). This suggests that increased vascular tone and impaired autoregulatory ability in postischemic AKI may be due, in part, to a loss of normal distensibility of the vasculature associated with disorganized actin filaments of smooth muscle cells.

Interestingly, all the recipients destined to have sustained AKI showed the most severe grade of damage to the peritubular capillary endothelium. This finding may suggest that maintaining endothelial integrity is critical to recovery of the graft function. The endothelium is the largest organ in the body. Its roles in vasodilation, controlling thrombotic tendency, and the initiation and maintenance of inflammation have been acknowledged (33). Brodsky et al. (6) demonstrated functional protection of ischemic kidneys by transplanting endothelial cells or surrogate cells expressing endothelial nitric oxide synthase into rats subjected to renal artery clamping. This finding suggested that endothelial cell dysfunction is the primary cause of the “no-reflow” phenomenon (6). We utilized vWF to assess the integrity of the endothelium since it is prevalent in endothelial cells and its circulating level has served as a marker of endothelial damage/dysfunction in various disease states such as atherosclerosis, hypertension, diabetes, chronic and acute renal failure, and systemic inflammatory response syndrome (5, 17, 24, 30). Staining endothelium with a different marker, anti-PECAM-1 (anti-CD31, Immunotech), has also been tried in our subjects. However, the intensity of the staining was increased in cadaveric allografts probably due to enhanced expression of PECAM after ischemia-reperfusion (data not shown), which made our semiquantitative
scoring of structural damage to the endothelium difficult and inaccurate. Therefore, we chose to use vWF staining for scoring of structural damage to endothelial cells. This study demonstrated that subjects destined to have recovery of their graft function had more preserved vWF immunostaining along the peritubular capillary endothelial lining compared with those destined to have sustained ARF. Underlying mechanism(s) explaining what triggered the recovery of endothelial cell integrity in subjects destined to have recovery or whether they had sustained less damage to the endothelium remains to be clarified. However, our finding suggests that intact peritubular capillary endothelium is beneficial to renal functional recovery and can in part predict recovery of the graft function.

Pericytes are multifunctional cells of microvessels that wrap around endothelial cells. In rabbits, pericytes cover the distal segments of the efferent arterioles, descending vasa recta, and peritubular capillaries (13, 29, 34). A single layer of smooth muscle cells of efferent arterioles extends 50–100 μm from the glomerulus, at which point there is an abrupt transition in cell type to that of a pericyte. Pericytes contain myofilaments and dense bodies, suggesting contractile capacity (13). Pallone et al. (28) reported the importance of descending vasa recta pericytes in the regulation of renal medullary blood flow. The interaction of endothelial cells and pericytes regulates each other’s mitotic rates and the tendencies to migrate into regions of hypoxia, as exemplified by angiogenesis, wound healing, and tumor growth. The process is mediated by many factors such as platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), angiogenin, angiopoietin-1, and angiopoietin-2 (35). Basile et al. (4) observed that a reduction in the peritubular capillary density following AKI results in a persistent reduction in renal Po2, thus contributing to the progression of chronic renal failure following AKI. A musclespecific anti-actin antibody reacts strongly with smooth muscle cells and pericytes but not endothelial cells (29, 15). Therefore, we used a monoclonal anti-α-smooth muscle actin antibody to identify pericytes, which may fall into a larger category of myofibroblast or myoepithelial cells, in the peritubular space. Increased peritubular interstitial α-smooth muscle actin staining was observed in cortical tissue sections from patients destined to recover their renal function and may indicate activation or proliferation of pericytes. This suggests that peritubular pericytes are playing a role in recovery of the graft function, possibly through the regulation of vasomotor tone and angiogenesis. Pericytes are known to be quiescent in the normal state but stimulated to proliferate on stress or in disease states such as ischemia (12, 27, 31, 37). α-Smooth muscle actin staining is known to increase in activated pericytes on stimulation (37). Of note, our control tissue also showed increased α-smooth muscle actin staining. The control tissues were obtained from healthy kidneys before an arterial clamp was applied to the kidney during nephrectomy for kidney donation.
or removal of renal cell cancer. There were ~2 h of operating time before the biopsies were performed. Therefore, our control tissues were actually from normal kidneys that had sustained a minor ischemic injury associated with vascular manipulation during the procedure. Our finding in the control tissues suggests that normal kidneys may be capable of maximally increasing pericytes even on a minor ischemic event.

In summary, we observed disorganization of F-actin filaments of vascular smooth muscle cells, disruption of endothelial cells, and increased peritubular interstitial pericytes in cadaveric renal allografts after ischemia-reperfusion. Endothelial integrity of peritubular capillaries was better preserved, and pericytes were more pronounced in patients destined to have recovery of their graft function compared with those destined to have sustained ARF. These findings suggest that damage to the smooth muscle actin cytoskeleton and endothelial cells is prevalent in renal vasculature after ischemia-reperfusion. Maintaining the integrity of the peritubular capillary endothelium and increasing pericytes may be essential to the recovery of renal function, implicating a contributory role of the microvascularity in initiating renal functional recovery in postischemic AKI/ATN.

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