Renal Microvascular Disease Determines the Responses to Revascularization in Experimental Renovascular Disease

Alejandro R. Chade, MD; Silvia Kelsen, BS

Background—Percutaneous transluminal renal angioplasty (PTRA) is the most frequent therapeutic approach to resolving renal artery stenosis (RAS). However, renal function recovers in only 30% of the cases. The causes of these poor outcomes are still unknown. We hypothesized that preserving the renal microcirculation distal to RAS will improve the responses to PTRA.

Methods and Results—RAS was induced in 28 pigs. In 14, vascular endothelial growth factor (VEGF)-165 0.05 μg/kg was infused intrarenally (RAS + VEGF). Single-kidney function was assessed in all pigs in vivo using ultrafast CT after 6 weeks. Observation of half of the RAS and RAS + VEGF pigs was completed. The other half underwent PTRA and repeated VEGF, and CT studies were repeated 4 weeks later. Pigs were then euthanized, the stenotic kidney removed, renal microvascular (MV) architecture reconstructed ex vivo using 3D micro-CT, and renal fibrosis quantified. The degree of RAS and hypertension were similar in RAS and RAS + VEGF. Renal function and MV density were decreased in RAS but improved in RAS + VEGF. PTRA largely resolved RAS, but the improvements of hypertension and renal function were greater in RAS + VEGF + PTRA than in RAS + PTRA, accompanied by a 34% increase in MV density and decreased fibrosis.

Conclusions—Preservation of the MV architecture and function in the stenotic kidney improved the responses to PTRA, indicating that renal MV integrity plays a role in determining the responses to PTRA. This study indicates that damage and early loss of renal MV is an important determinant of the progression of renal injury in RAS and instigates often irreversible damage. (Circ Cardiovasc Interv. 2010;3:00-00.)

Key Words: renal artery obstruction ■ microsurgical revascularization ■ 3D imaging ■ renal circulation

Renal artery stenosis (RAS) is a predictor of major cardiovascular events, adverse renal outcomes, and mortality,1 independent of other prevalent cardiovascular risk factors. RAS is present in up to 40% of patients with coronary or peripheral atherosclerotic vascular disease.2 This increase in prevalence is paralleled by a growing use of renal artery interventions such as percutaneous transluminal renal angioplasty (PTRA), the most frequent therapeutic approach to treat RAS that has increased dramatically during the past 2 decades.3 The technical advances and increase in success rates (almost 100%) have been the impetus of the growing use of PTRA to treat RAS. However, the outcomes of this intervention are far from optimal because resolution of hypertension is not obtained, and renal function does not recover or becomes even further aggravated in up to 70% of the cases5 despite successful resolution of the stenosis and restoration of renal blood flow. The reasons for these relatively poor outcomes are still unknown.

Clinical Perspective on p ●●●

Functional and structural abnormalities in the microvasculature of the stenotic kidney contribute to the pathophysiology of ischemic renal injury. Microvascular (MV) remodeling, damage, and loss have been observed in several experimental models of both acute and chronic ischemic renal injury, underscoring the important role renal MV disease plays in the progression of renal damage.5,7 However, whether a deterioration of the renal microcirculation in the chronically stenotic kidney plays a role in defining the renal outcomes in response to revascularization has never been investigated. We have previously shown in a model of chronic RAS that mimics early chronic human renovascular disease that the stenotic kidney develops MV rarefaction as the disease evolves, which is paralleled by a progressive deterioration of renal function, extensive renal fibrosis, and a significant decrease in renal bioavailability of the central angiogenic factor vascular endothelial growth factor (VEGF),8 a key player in maintaining the renal MV integrity and promoting MV proliferation and repair. In addition, we have shown recently that deleterious changes were largely attenuated by preserving renal VEGF bioavailability9 in the stenotic kidney. Decreases in VEGF availability and activity have been shown to play a role in triggering renal injury in different physio-
Sustained effect to increase the collateral MV density and perfusion of the ischemic myocardium.\textsuperscript{16-17} Throughout the 6 weeks following the induction of RAS, blood pressure was continuously monitored through a telemetry system implanted at baseline in the right femoral artery. Mean arterial pressure was recorded at 5-minute intervals and averaged for each 24-hour period.\textsuperscript{14,15} Other animals were used as normal controls (n=14).

At 6 weeks after induction of RAS, all the pigs underwent renal angiography to quantify the degree of RAS. The pigs were anesthetized with IM telazol 5 mg/kg and xylazine 2 mg/kg, intubated, and mechanically ventilated on room air. Anesthesia was maintained with a mixture of ketamine 0.2 mg/kg per min and xylazine 0.03 mg/kg per min in normal saline administered through an ear vein cannula at a rate of 0.05 mL/kg per min. Under sterile conditions and fluoroscopic guidance, an 8-F arterial catheter was advanced to the renal artery proximal to the stenosis, and renal angiography was performed, as previously described.\textsuperscript{14,15,18} Extent of the stenosis was assessed as the decrease in luminal diameter of the renal artery at the most stenotic point compared to a proximal stenosis-free segment. After angiography, the catheter was positioned in the superior vena cava, and in vivo helical multidetector CT (MDCT) flow studies were performed. In brief, sequential acquisition of 160 consecutive scans were obtained after a central venous injection of iopamidol 0.5 mL/kg per 2 s for assessment of single-kidney renal blood flow (RBF) (mL/min), perfusion (mL/min per g tissue), and glomerular filtration rate (GFR) (mL/min), as previously detailed and validated.\textsuperscript{14,19,20} Studies were repeated during suprarenal infusion of the prototypical endothelium-dependent vasodilator acetylcholine at a rate of 5 μg/kg per min to test intrarenal endothelial function. Renal vascular resistance was calculated by dividing the mean arterial pressure (at the moment of the in vivo studies) and MDCT-derived RBF.

Half of the animals were euthanized after completion of the in vivo studies at 6 weeks. On completion of the MDCT studies and while still under anesthesia, all the remaining RAS and RAS+VEGF animals underwent PTRA under fluoroscopic guidance using a balloon catheter plus tantalum stent deployment to optimize vascular patency for revascularization. In brief, a 7 mm×1 cm PTCA balloon catheter was engaged in the stenotic renal artery and inflated for 30 seconds at 10 atm and a few minutes later again at 14 atm to fully dilate the stenosis. Then a standard tantalum stent matched to the size of the renal artery and length of stenosis (usually a few mm) was implanted in the renal artery following balloon dilatation. Administration of intrarenal VEGF 0.05 μg/kg was repeated in the pigs that received VEGF at the induction of RAS. Blood pressure was continuously monitored by telemetry, and all the pigs were observed for 4 additional weeks before undergoing renal angiography to determine the effects of PTRA on the renal artery, followed by in vivo basal and stimulated MDCT in vivo studies at 6 weeks to determine the effects of PTRA on renal hemodynamics and function.

After completion of all the in vivo studies (at 6 weeks and at 10 weeks) (Figure 1), the pigs were allowed to recover for 2 days to allow for contrast media washout and then were euthanized with IV sodium pentobarbital 100 mg/kg. The kidneys were removed using a retroperitoneal incision and immersed in heparinized saline 10 U/mL. A lobe of tissue was used for micro-CT reconstruction, whereas another lobe of tissue was removed from one end of the kidney, snap-frozen in liquid nitrogen, and stored at −80°C to quantify mRNA expression of VEGF receptors Flt-1 and Flk-1 by RT-PCR\textsuperscript{2} or preserved in 10% formalin to later perform immuno-histochemistry against CD31\textsuperscript{19} and investigate renal morphology in midhilar renal cross-sections stained with trichrome.\textsuperscript{14}

### MDCT Analysis

Manually traced regions of interest were selected in MDCT images of the aorta, renal cortex, medulla, and papilla and their densities sampled. Time-density curves were generated and fitted with extended γ-variate curve-fits, and the area enclosed under each segment of the curve and its first moment were calculated using the curve-fitting parameters. These were used to calculate single-kidney
RBF (mL/min), GFR (mL/min), and renal perfusion (mL/min per g tissue) through previously validated methods.14,19,20

Micro-CT
The stenotic kidney was perfused under physiological perfusion pressure with an intravascular contrast agent. The kidney samples were scanned at 0.3° increments using a micro-CT scanner and reconstructed at 9-μm resolution for subsequent analysis, as previously described.8,9,21 Images were analyzed with the Analyze software package. The cortex was tomographically divided into 12 levels starting at the juxtamedullary cortex obtained at equal intervals, and the spatial density and distribution of microvessels (diameters 10 to 500 μm) were calculated.9

Renal VEGF
Renal protein concentration of VEGF in the stenotic kidney was measured in tissue homogenates using an enzyme-linked immunoassay.9

Immunohistochemistry
Because of their size, capillaries (microvessels <10 μm) cannot be identified by the micro-CT technique. Therefore, peritubular and glomerular capillaries were quantified in 5-μm paraffin-embedded midhilar renal cross-sections to assess the expression of CD31 (1:80). The secondary antibody, IgG Envision Plus, was followed by staining with the Vector NovaRED substrate kit according to vendor instructions.

Histology
Midhilar 5-μm cross-sections of each kidney (1 per animal) were examined using a computer-aided image-analysis program. In each representative slide, trichrome staining was semiautomatically quantified in 15 to 20 fields by the computer program and expressed as a percentage of staining of total surface area, and the results from all fields were averaged.14 Glomerular score was assessed by recording the number of sclerotic glomeruli out of 100 counted glomeruli, as previously described.14 In addition, to quantify CD31 immunoreactivity, randomly selected 15 to 20 visual fields from each sample (1 slide per animal) were analyzed at ×40 magnification. Capillaries then were identified as vessels at approximately 8 to 10 μm in diameter constituted of a single layer of endothelial cells and quantified as the number of capillaries per visual field, as recently described.9

Statistical Analysis
Results are expressed as mean±SEM. Comparisons within groups were performed using paired Student t test and among groups using 1-way ANOVA with Fisher least significant difference post hoc tests for correction for multiple comparisons. Statistical significance was accepted for \( P<0.05 \). For data measured over time (blood pressure) a 2-way repeated-measures ANOVA was used, and statistical significance was accepted for \( P\leq0.05 \).

Results
Pre-PTRA
The degree of stenosis and hypertension were similar in RAS and RAS+VEGF animals after 6 weeks (73.2±5.8% and 71.6±7.7% and 139.5±3.0 mm Hg and 147.4±6.0 mm Hg, respectively; \( P<0.05 \)) versus normal controls before PTRA. RBF, perfusion, GFR (Figure 2, top), cortical MV and capillary density, and renal VEGF (Figure 2, middle and bottom) were significantly decreased in RAS but improved in RAS+VEGF and accompanied by a significant attenuation in renal vascular resistance, glomerulosclerosis, and tubulointerstitial fibrosis, as we have shown previously.9

Post-PTRA
PTRA largely resolved the stenosis in all animals, and no significant residual stenosis was observed 4 weeks after
PTRA in any of the RAS or RAS/VEGF pigs (Figure 3). However, a modest decrease in blood pressure was observed in RAS+PTRA, whereas in the RAS+VEGF+PTRA pigs, blood pressure decreased to a larger extent and almost returned to basal levels (Figure 4A). This was accompanied by a more accentuated decrease in renal vascular resistance compared with normal controls in RAS+VEGF+PTRA (0.21±0.02 mm Hg/mL per min; P<0.04) and RAS+PTRA (0.25±0.01 mm Hg/mL per min; P<0.07). Furthermore, PTRA combined with VEGF administration dramatically improved RBF, GFR, and regional perfusion (Figure 4B) and restored MV endothelial function (Figure 4C, ANOVA P<0.05 for all). These effects were accompanied by a 3-fold increase in the mRNA expression of the VEGF receptor Flk-1, a 34% increase in MV density in RAS+VEGF compared to RAS (Figure 5), and decreased glomerulosclerosis and tubulointerstitial fibrosis (Figure 6).

**Discussion**

The current study extends our previous findings and highlights the central role renal MV disease plays not only in the progression of renal injury, but also in the responses to renal revascularization. The stenotic kidney shows significant MV rarefaction accompanied by a marked deterioration of renal function and increased fibrosis. Preservation of the renal MV architecture and function largely improved the function of the
stenotic kidney. Furthermore, to our knowledge, this study shows for the first time that targeting the renal microcirculation in the stenotic kidney dramatically improves the renal functional responses to PTRA, indicating that renal MV rarefaction (and consequently the extent of renal damage) plays a critical role in determining the outcomes of revascularization. Moreover, this study indicates that the damage and early loss of the renal microvessels (as observed after 6 weeks of RAS) and the deterioration of the renal angiogenic response (as suggested by decreased VEGF and downstream mediators) are important determinants for the progression of renal injury and likely demarcate the point of often irreversible damage in the stenotic kidney.

Catheter-based therapy for hemodynamically significant RAS is the preferred method of revascularization. The use of this intervention has been consistently growing for the past 20 years, with tremendous progress in successfully restoring the blood flow through a previously stenotic renal artery (>95% of the cases). However, it is disconcerting that resolution of hypertension and, mainly, improvements in renal function are still at best modest, with improvement rates of around 30% of the cases in most reports. The reasons for this discordance

Figure 5. Representative 3D micro-CT reconstruction of the renal MV architecture after PTRA. RAS+VEGF+PTRA showed a 34% increase in MV density compared to RAS+PTRA.

Figure 6. Renal fibrosis and quantification in normal, RAS+PTRA, and RAS+VEGF+PTRA kidneys. Individual representative glomeruli and tubulointerstitial regions are shown as examples to illustrate the quantitative information. VEGF+PTRA decreased the damage of the stenotic kidney compared to PTRA alone. *P<0.05 versus normal controls. †P<0.05 versus RAS.
between the success rate and outcomes are still unknown and have been the topic of numerous analyses and discussions but without a definitive answer. White and Olin recently published an extensive in-depth review where they postulated that the reasons for these relatively poor outcomes may be the result of a combined poor selection of the patients; poor discrimination of the severity of the lesions; and, most importantly, insufficient assessment of the injury of the renal parenchyma distal to the stenosis. Therefore, whether the decrease in MV density is likely a central event that determines the functional and structural deterioration of the stenotic kidney. Nevertheless, whether this reasoning partly explains the poor responses of the stenotic kidney to revascularization has never been investigated or established.

The current study attempted to elucidate the role of MV disease by testing the hypothesis that changes in the renal MV architecture and function are crucial for the progression of renal injury and, mainly, for the renal responses to revascularization. We use a well-established model of RAS: a surrogate of early chronic renovascular disease that results in the development of a hemodynamically significant stenosis, leading to hypertension and significant renal functional and structural injury as early as after 4 to 6 weeks. This model resembles human disease in several ways because RAS gradually develops and involves progressive vascular wall injury, as occurs in human RAS, thus constituting a clinically relevant model of renovascular disease. In addition, being a large-animal model, it offers a unique opportunity to perform surgical and pharmacological interventions in a manner that potentially could be applicable to humans as well as allows us to determine the effects of the disease and responses to interventions accurately. We have shown that the renal functional deterioration in our model is accompanied by reduced renal VEGF and MV density. By infusing VEGF intrarenally at the onset of the stenosis, we have shown recently that this intervention improved MV density and function and decreased fibrosis without modifying the degree of RAS or hypertension, suggesting a targeted effect into the stenotic renal parenchyma. Previous studies have shown sustained long-term effects of VEGF in improving tissue perfusion and development of new vessels. We observed sustained beneficial effects of a single intrarenal infusion of VEGF, which were reflected not only by the improvements in renal function and preservation of the cortical and medullary MV architecture, but also by the restoration of downstream mediators of VEGF, such as angiopoietins and endothelial nitric oxide synthase. Additionally, the augmented VEGF bioavailability in the RAS + VEGF-treated kidneys facing an enhanced expression of the VEGF receptor Flk-1 may have contributed to a more organized vascular proliferative response and MV function by this approach. It is also possible that a reduction in renal fibrosis in turn may have resulted in preservation of the sources of VEGF, further contributing to maintaining the angiogenic cascade in the stenotic kidney in response to an ischemic insult. In addition to mediating renal MV proliferation and repair, VEGF can exert renoprotection by stimulating proliferation and survival of renal epithelial cells, which in turn can further attract endothelial cells to promote vasculogenesis in a complex autocrine/paracrine mechanism.

Because catheter-based revascularization is the most frequent therapeutic approach to treating RAS in humans, all RAS and RAS + VEGF pigs underwent PTRA, and a stent was deployed to assure vascular patency. Interestingly, despite complete restoration of blood flow and resolution of the hemodynamically significant stenosis, blood pressure and renal vascular resistance decreased less in the RAS pigs compared to RAS + VEGF, where both decreased to a level that was not different compared to normal time controls. The more modest decrease of these parameters in RAS post-PTRA argues against a pure renovascular origin of hypertension in this model, which likely mimics what occurs in humans. Indeed, clinical data show that resolution of hypertension after successful revascularization is observed in <10% of patients, which possibly is weighted on the degree of renal damage as an important contributor to hypertension. In addition, another study suggested that the GFR pre-PTRA may anticipate whether the outcomes of revascularization will affect renal function enhancement and hypertension control. The significant preservation of renal function and attenuation of fibrosis and MV damage and loss pre-PTRA in RAS + VEGF animals (despite similar degree of RAS) likely played a role for the significant improvement in RBF, GFR, and regional perfusion and in restoring MV endothelial function post-PTRA unlike in untreated RAS where renal function remained attenuated. Importantly and supporting our hypothesis, these beneficial effects were accompanied by a 34% increase in MV density in VEGF + PTRA compared to PTRA alone and decreased fibrosis. Moreover, the significant improvement in the MV responses to endothelium-dependent challenge with acetylcholine in VEGF-treated pigs indicates that this intervention resulted in not only more, but also better functional new vessels.

A limitation of this study is that most of these effects were largely preventive because VEGF was administered at the induction of RAS. Hence, further studies testing this or similar targeted interventions on renal microvessels after established renal injury (eg, administering VEGF after RAS and renal injury develop), in combination with renoprotective drugs frequently used in patients with renovascular disease (eg, angiotensin-receptor blockers, statins), and at a later stage of the disease will cement the role that protection of renal MV integrity plays in defining the fate of the stenotic
kidney and its outcomes after catheter-based interventions. Furthermore, we cannot rule out the possibility of VEGF achieving beneficial effects on the stenotic kidney independently of MV improvements by directly downregulating other injurious mechanisms, such as fibrosis or apoptosis. We are aware that another limitation is that the study design is not yet directly clinically applicable. However, the results of our studies challenge current therapies in human renovascular disease and strongly imply a link between renal MV architecture and function and progression of renal injury because the degree of RAS and hypertension was similar in all RAS animals, but prevention of MV rarefaction preserved the hemodynamics and function and improved the responses to PTRA.

In conclusion, our findings support a novel concept and may constitute the first step to unravel the complex mechanisms that determine the recovery of the stenotic kidney in humans. By refining the timing of PTRA, the most frequent and established therapeutic approach to treat patients with RAS, and determining whether combining PTRA with a targeted intervention deemed to protect the renal microcirculation is feasible, our studies could potentially open new avenues to improve the treatment of patients with chronic renovascular disease.

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Disclosures
None.

References
Percutaneous transluminal renal angioplasty with stenting is technically effective for treating renal artery stenosis; however, the ischemic kidney does not improve or deteriorates in 60% to 70% of patients undergoing these treatments. The reasons behind these relatively poor outcomes are still unknown, but we have previously shown that the stenotic kidney undergoes a progressive deterioration of its function paralleled by an evolving intrarenal microvascular damage and loss. Using a well-established swine model of chronic renal artery stenosis, we tested the hypothesis that preserving the renal microcirculation in the stenotic kidney will improve the responses to revascularization. We observed that preservation of the renal microvascular architecture and function improved the outcomes of percutaneous transluminal renal angiography, supporting a crucial role of renal microvascular integrity in determining the progression of renal injury in renal artery stenosis and the responses to revascularization.
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