Endothelial Outgrowth Cells Shift Macrophage Phenotype and Improve Kidney Viability in Swine Renal Artery Stenosis


Objective—Endothelial outgrowth cells (EOC) decrease inflammation and improve endothelial repair. Inflammation aggravates kidney injury in renal artery stenosis (RAS), and may account for its persistence upon revascularization. We hypothesized that EOC would decrease inflammatory (M1) macrophages and improve renal recovery in RAS.

Approach and Results—Pigs with 10 weeks of RAS were studied 4 weeks after percutaneous transluminal renal angioplasty (PTRA+stenting) or sham, with or without adjunct intrarenal delivery of autologous EOC (10×10⁶), and compared with similarly treated normal controls (n=7 each). Single-kidney function, microvascular and tissue remodeling, inflammation, oxidative stress, and fibrosis were evaluated. Four weeks after PTRA, EOC were engrafted in injected RAS-kidneys. Stenotic-kidney glomerular filtration rate was restored in RAS+EOC, RAS+PTRA, and RAS+PTRA+EOC pigs, whereas stenotic-kidney blood flow and angiogenesis were improved and fibrosis attenuated only in EOC-treated pigs. Furthermore, EOC increased cell proliferation and decreased the ratio of M1 (inflammatory)/M2 (reparative) macrophages, as well as circulating levels and stenotic-kidney release of inflammatory cytokines. Cultured-EOC released microvesicles in vitro and induced phenotypic switch (M1-to-M2) in cultured monocytes, which was inhibited by vascular endothelial growth factor blockade. Finally, a single intrarenal injection of rh-vascular endothelial growth factor (0.05 μg/kg) in 7 additional RAS pigs also restored M1/M2 ratio 4 weeks later.

Conclusions—Intrarenal infusion of EOC after PTRA induced a vascular endothelial growth factor–mediated attenuation in macrophages inflammatory phenotype, preserved microvascular architecture and function, and decreased inflammation and fibrosis in the stenotic kidney, suggesting a novel mechanism and therapeutic potential for adjunctive EOC delivery in experimental RAS to improve PTRA outcomes. (Arterioscler Thromb Vasc Biol. 2013;33:1006-1013.)

Key Words: kidney hypertension • macrophages • progenitor cells • renal artery stenosis • revascularization

Renal artery stenosis (RAS) produces chronic underperfusion of the renal parenchyma, which is associated with development of chronic kidney disease, renovascular hypertension, cardiovascular disease, and cardiac events. Furthermore, progressive renal injury often fails to regress upon restoration of vessel patency by percutaneous transluminal renal angioplasty (PTRA). These adverse outcomes are likely secondary to lingering kidney tissue injury that remains unresolved by restoring blood flow alone. Indeed, we have shown that PTRA-alone improves glomerular filtration rate (GFR) and renal endothelial function, but fails to fully resolve tissue injury in the stenotic swine kidney. Renal inflammation, oxidative stress, tubulointerstitial injury, and microvascular rarefaction all persist after revascularization. These observations underscore the need for more effective strategies to restore integrity and decrease the progression to fibrosis in the stenotic kidney.

Mounting data highlight inflammatory cells as important mediators of irreversible kidney damage, which may contribute to the limited response to revascularization observed in renovascular hypertension. We have previously shown in experimental renovascular disease increased stenotic-kidney expression of the proinflammatory cytokite monocyte chemotactant protein (MCP-1), accompanied by macrophage infiltration and microvascular rarefaction. Moreover, MCP-1 blockade markedly decreases inflammation and fibrosis and increases microvascular density, uncovering the potential of
modulating inflammatory infiltration to preserve the kidney in chronic RAS.

Importantly, 2 different subpopulations of macrophages may accumulate in the injured kidney after acute ischemic injury. Initially, there is an influx of proinflammatory (M1) macrophages characterized by the expression of inducible nitric oxide synthase (iNOS), release of inflammatory markers like tumor necrosis factor (TNF)-α, and upregulation of nuclear factor κB. The subsequent healing process is associated with increased abundance of macrophages showing an alternative reparative noninflammatory (M2) phenotype, characterized by expression of mannose receptor, arginase (Arg)-1, or interleukin (IL)-10. Conceivably, a change in the relative preponderance of these 2 macrophage subpopulations might determine the balance between renal injury and repair. However, the characteristics of renal macrophages in chronic ischemia, and whether reducing tissue inflammation and the M1/M2 ratio in the stenotic kidney might be associated with better outcomes after revascularization remains unknown.

Our group has previously demonstrated that intrarenal delivery of endothelial outgrowth cells (EOC) elicits neovascularization and mitigates renal injury in swine RAS9,10 and that the stenotic human kidney releases inflammatory cytokines that correlate with EOC retention, implicating kidney inflammation in progenitor cell recruitment beyond the stenotic lesion.11 However, the effects of EOC delivery on kidney inflammation, and whether EOC are capable of improving renal structural and functional recovery after PTRA, remain to be elucidated. Therefore, this study tested the hypothesis that adjunct intrarenal infusion of autologous EOC at the time of revascularization would attenuate renal inflammation and cellular injury and improve renal recovery in swine RAS. Moreover, we hypothesized that this might be associated with a decreased prevalence of M1 relative to M2 macrophages.

Materials and Methods

Materials and Methods are available in the online-only Supplement.

Results

At 6 weeks after induction and before revascularization (Figure I in the online-only Data Supplement), RAS was significant (72.4±3.2%; P<0.09 among RAS groups) and mean arterial pressure similarly elevated in all RAS animals (P<0.01 in all compared with normal).

The Table summarizes the systemic characteristics, renal hemodynamics, and function 4 weeks after PTRA or sham. Serum creatinine levels were similarly elevated in RAS, RAS+EOC, and RAS+PTRA compared with normal (P=0.003, 0.007, and 0.02, respectively), but normalized in RAS+PTRA+EOC-treated pigs (P=0.19 versus normal). As common in chronic RAS, plasma renin activity was similar among the groups.

Revascularization was successful in all 14 pigs, with no residual stenosis observed 4 weeks later; mean arterial pressure returned to baseline levels shortly after PTRA (P>0.05 versus RAS, P>0.05 versus normal; Table and Figures II and IIIA in the online-only Data Supplement).

In Vivo Studies

EOC Characterization and Functionality

As previously demonstrated,7 the number of colony forming units did not differ between RAS and normal pigs. Early EOC initially expressed CD14 and CD133, which were downregulated in late EOC, whereas late EOC expressed kinase-insert

Table. Systemic Characteristics and Renal Hemodynamics (Mean±SEM) in Normal Controls or Pigs With Renal Artery Stenosis (RAS), Untreated or Treated With EOC (n=7 Each) 4 Weeks After PTRA or Sham

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>Normal+EOC</th>
<th>RAS</th>
<th>RAS+EOC</th>
<th>RAS+PTRA</th>
<th>RAS+PTRA+EOC</th>
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</thead>
<tbody>
<tr>
<td>Body weight, Kg</td>
<td>52.6±7.9</td>
<td>45.6±8.9</td>
<td>49.1±4.1</td>
<td>50.4±6.4</td>
<td>45.3±5.7</td>
<td>51.4±5.4</td>
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<tr>
<td>Degree of stenosis, %</td>
<td>0±0</td>
<td>0±0</td>
<td>71.7±27.8#</td>
<td>71.4±5.6#</td>
<td>0±0‡</td>
<td>0±0‡</td>
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<tr>
<td>Mean arterial pressure, mm Hg</td>
<td>103.4±10.4</td>
<td>102.3±11.2</td>
<td>142±15.5#</td>
<td>148.9±14.2#</td>
<td>106.3±21.8‡</td>
<td>105.6±18.2‡</td>
</tr>
<tr>
<td>Serum creatinine, mg/dL</td>
<td>1.3±0.1</td>
<td>1.4±0.3</td>
<td>1.9±0.1#</td>
<td>1.9±0.2#</td>
<td>1.7±0.1*</td>
<td>1.5±0.1†</td>
</tr>
<tr>
<td>PRA, ng/mL per h</td>
<td>0.22±0.05</td>
<td>0.21±0.03</td>
<td>0.18±0.05</td>
<td>0.19±0.03</td>
<td>0.19±0.04</td>
<td>0.20±0.03</td>
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<tr>
<td>Cortical volume, cc</td>
<td>109.6±12.0</td>
<td>112.8±10.8</td>
<td>57.3±18.0#</td>
<td>94.4±17.2†</td>
<td>98.2±7.1‡</td>
<td>100.9±5.5†</td>
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<td>Medullary volume, cc</td>
<td>40.6±7.1</td>
<td>41.7±6.2</td>
<td>20.9±5.4#</td>
<td>23.8±5.8#</td>
<td>24.7±4.8#</td>
<td>33.3±4.6‡</td>
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<td>Cortical perfusion, mL/min per cc</td>
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<td>4.8±0.7</td>
<td>3.1±0.9#</td>
<td>4.5±1.0#</td>
<td>3.6±0.5#</td>
<td>4.5±1.0#</td>
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<tr>
<td>Medullary perfusion, mL/min per cc</td>
<td>2.9±0.5</td>
<td>2.8±0.9</td>
<td>2.3±0.7</td>
<td>2.5±0.9</td>
<td>2.7±0.4</td>
<td>2.8±0.9</td>
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<tr>
<td>RBF, mL/min</td>
<td>580.7±30.5</td>
<td>596.9±46.8</td>
<td>315.8±62.5#</td>
<td>484.4±44.2†</td>
<td>386.0±17.4*‡</td>
<td>521.8±50.1†</td>
</tr>
<tr>
<td>RBF response to Ach, mL/min</td>
<td>747.0±24.5†</td>
<td>729.9±46.1†</td>
<td>317.2±70.9#</td>
<td>562.4±59.5‡†</td>
<td>437.4±40.1*††</td>
<td>611.2±30.3‡†</td>
</tr>
<tr>
<td>GFR, mL/min</td>
<td>82.9±13.3</td>
<td>95.5±10.4</td>
<td>45.2±9.7#</td>
<td>68.5±7.9‡</td>
<td>72.5±4.3‡</td>
<td>79.6±5.5‡</td>
</tr>
<tr>
<td>GFR response to Ach, mL/min</td>
<td>103.9±21.0</td>
<td>115.8±21.8</td>
<td>46.8±9.1#</td>
<td>79.1±5.9†</td>
<td>83.0±5.1†</td>
<td>90.0±7.2†</td>
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<tr>
<td>Urine isoprostane, pg/mL</td>
<td>264.9±113.5</td>
<td>188.5±134.9</td>
<td>344.2±307.6</td>
<td>297.9±314.7</td>
<td>190.7±96.8</td>
<td>274.1±158.7</td>
</tr>
</tbody>
</table>

Ach indicates acetylcholine; EOC, endothelial outgrowth cells; GFR, glomerular filtration rate; PRA, plasma renin activity; PTRA, percutaneous transluminal renal angioplasty; RAS, renal artery stenosis; and RBF, renal blood flow.

*P<0.05 vs normal.
#P<0.05 vs RAS+PTRA+EOC.
†P<0.05 vs RAS.
‡P<0.05 vs baseline.
domain receptor and CD34, suggestive of endothelial differentiation (data not shown).

Four weeks after intraarterial administration, EOC retention rate was 13% to 16% in RAS and 3% to 4% in normal animals (because of homing and adherence in ischemic kidneys).\textsuperscript{9,13} Injected EOC co-stained with proliferating cell nuclear antigen (Figure IVA in the online-only Data Supplement) and incorporated into CD31+ blood vessels (Figure IVB in the online-only Data Supplement) or into tubules\textsuperscript{9} (Figure IIB in the online-only Data Supplement). Vascular endothelial growth factor (VEGF) immunoreactivity was elevated compared with controls, particularly in the vicinity of CD34-stained progenitors (Figure VA and VB in the online-only Data Supplement), suggesting paracrine effects.

Renal Hemodynamics and Function
Basal stenotic-kidney cortical perfusion and renal blood flow were similarly lower in RAS and RAS+PTRA compared with normal (Table), but not in RAS+EOC and RAS+PTRA+EOC. However, basal GFR, cortical perfusion, renal blood flow, and GFR responses to acetylcholine were all similar to normal in RAS+PTRA, RAS+EOC, and RAS+PTRA+EOC-treated pigs.

Renal Injury Mechanisms
Microvascular and interstitial remodeling: Spatial density of cortical microvessels was similarly diminished in RAS and RAS+PTRA, but preserved in both EOC-treated groups (Figure 1A and B). Moreover, microvascular diameter was higher in RAS and RAS+PTRA compared with normal, implying relative loss of small vessels, but not in EOC-treated pigs (Figure 1C). Vessel tortuosity, which characterizes angiogenic vessels, was similarly increased in RAS and RAS+PTRA, but again preserved in RAS+EOC and RAS+PTRA+EOC pigs (Figure 1D). Renal expression of VEGF was lower than normal in RAS, and higher than RAS only in animals treated with both PTRA+EOC (Figure 1E), whereas VEGF receptor-2 expression was higher than RAS in all treated groups. Renal basic fibroblast growth factor expression was similarly blunted in RAS and RAS+EOC, but restored in RAS+PTRA.
and RAS+PTRA+EOC. Expression of endothelial nitric oxide synthase was downregulated in RAS, but higher than RAS in RAS+PTRA and RAS+PTRA+EOC pigs (Figure 1E).

**Cell proliferation or loss:** Proliferating cell nuclear antigen immunoreactivity was ameliorated in RAS compared with normal. PTRA-alone restored it to normal levels and EOC-treatment to levels higher than normal in all pigs (Figure VI in the online-only Data Supplement). In all groups, proliferating cell nuclear antigen expression was equally distributed among glomerular, tubular, interstitial, and vascular compartments. The number of both terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) and caspase-3 apoptotic cells was higher than normal in stenotic-kidneys. RAS+PTRA-alone presented normal number of TUNEL+ cells, although the number of caspase-3+ cells remained elevated. However, animals treated with EOC (with or without PTRA) showed normal levels of both TUNEL+ and caspase-3+ cells. Renal expression of the proapoptotic B-cell lymphoma (Bcl)-associated X-protein (Bax) was elevated in RAS, but different from normal and RAS in RAS+EOC and RAS+PTRA, but was lower than RAS only in RAS+PTRA+EOC. Contrarily, expression of the antiapoptotic Bcl-xl was similar among the groups. Consequently, Bax/Bcl-xl ratio was higher than normal in RAS, RAS+EOC, and RAS+PTRA, but restored to normal levels in RAS+PTRA+EOC (Figure 1G).

**Oxidative stress:** Urine isoprostane levels were similar among the groups (Table). However, in situ production of superoxide anion in the poststenotic kidney was similarly higher in RAS and RAS+PTRA compared with normal, but decreased in EOC-treated pigs (Figure 1H and Figure VIIB in the online-only Data Supplement). Renal expression of p47 was upregulated in RAS, but not different from normal in RAS+EOC, RAS+PTRA, or RAS+PTRA+EOC animals. However, nitrotyrosine expression was elevated in RAS, but declined only in RAS+PTRA+EOC (Figure 1I).

**Inflammation:** CD68+/iNOS+ (M1) macrophages were abundant in all RAS groups regardless of treatment (Figure 2A and Figure VIIIA in the online-only Data Supplement), whereas CD68+/Arg-1+ (M2) macrophage count was elevated in all groups. Quantification of monocyte chemoattractant protein (MCP)-1 (C), interferon (IF)-γ (D), and tumor necrosis factor (TNF)-α (E) decreased only in EOC-treated groups. Quantification of fibronectin and collagen IV immunostaining (F). Quantification of stenotic-kidney tubulointerstitial fibrosis (G) and glomerular score (H). Renal protein expression of tissue inhibitor of metalloproteinases (TIMP)-2, transforming growth factor (TGF)-β, and plasminogen activator-inhibitor (PAI)-1 (I). *P<0.05 vs normal; †P<0.05 vs renal artery stenosis (RAS)+percutaneous transluminal renal angioplasty (PTRA)+endothelial outgrowth cells (EOC); and ‡P<0.05 vs RAS.
EOC-treated pigs (P<0.05 versus normal). Consequently, the elevated M1/M2 ratio observed in RAS, RAS+EOC, and RAS+PTRA was restored to normal levels in RAS+PTRA+EOC (Figure 2B). Systemic MCP-1 levels were similarly higher in RAS and RAS+PTRA compared with normal, but not in EOC-treated animals (Figure 2C), as were interferon-γ and TNF-α levels (Figure 2D and 2E). Furthermore, stenotic-kidney release of interferon-γ, MCP-1, and TNF-α was elevated in RAS and RAS+PTRA, but not different from normal levels in EOC-treated animals (Figure IXB–IXD in the online-only Data Supplement). Contrarily, renal release of the anti-inflammatory IL-10 was lower in RAS and RAS+PTRA, and normalized in RAS+EOC and RAS+PTRA+EOC animals (Figure IXE in the online-only Data Supplement).

Renal fibrosis: Fibronectin and collagen IV content (Figure 2F and Figure XA in the online-only Data Supplement), as well as tubulointerstitial fibrosis (Figure 2G and Figure XB in the online-only Data Supplement) and glomerular score (Figure 2H) were equally higher in RAS and RAS+PTRA compared with normal, but were lower than RAS in EOC-treated animals, whereas renal expression of tissue inhibitor of metalloproteinase-2 (Figure 2I) was similarly higher in all RAS groups compared with normal. Transforming growth factor (TGF)-β expression was also upregulated in RAS groups, but markedly downregulated in RAS+PTRA+EOC-treated pigs. Plasminogen activator inhibitor-1 expression decreased exclusively in RAS+PTRA+EOC pigs (Figure 2I).

Mechanisms of EOC Effects

Macrophage Activation In Vitro
EOC co-cultured with cultured polarized monocytes decreased protein expression of M1 markers (iNOS, TNF-α, and nuclear factor κB), and increased expression of M2 markers (IL-10 and Arg-1), suggesting a phenotype switch of macrophage function. Blockade of VEGF in EOC increased the expression of iNOS and TNF-α to levels higher than untreated activated monocytes, whereas nuclear factor κB expression was unaffected. VEGF blockade did not affect IL-10, but decreased Arg-1 expression. Conversely, IL-10 silencing did not affect expression of M1 or M2 markers on polarized monocytes, except for IL-10 expression that significantly declined (Figure VIIIIB in the online-only Data Supplement).

EOC-Derived Microvesicles
Cultured-EOC released multiple microvesicles expressing α4-integrin, β1-integrin, CD34, CD42b, CD14, p-selectin, CD34, and human leukocyte antigen class-I (Figure XI in the online-only Data Supplement).

Effect of VEGF on Macrophage Phenotype In Vivo
Direct intrarenal VEGF delivery decreased the number of M1 (CD68+/iNOS+) macrophages compared with RAS. Although the number of M2 (CD68+/Arg-1+) macrophages was unchanged, M1/M2 ratio was normalized in RAS+VEGF pigs (Figure XIIA– XIIC in the online-only Data Supplement).

Comparison of the Therapeutic Regimens
Table I in the online-only Data Supplement compares qualitatively the interventional strategies used to treat RAS (EOC and PTRA) alone and in combination. Although several parameters were lower in either EOC or PTRA-alone–treated animals, adjunctive EOC delivery during revascularization achieved the greatest reduction of both renal damage and hypertension in RAS.

Discussion
This study demonstrates that EOC induced a shift of renal macrophages from an inflammatory (M1) to reparative (M2) phenotype, evident both in vivo in chronic renal ischemia and in vitro in cultured monocytes. This transition could be blocked in vitro by inhibitors of VEGF. Furthermore, combining intrarenal delivery of autologous EOC with revascularization decreased the M1/M2 macrophage ratio and improved stenotic-kidney hemodynamics, function, and microvascular remodeling 4 weeks later. Importantly, the combination of PTRA+EOC decreased serum creatinine levels more effectively than PTRA-alone. Thus, this study revealed immunomodulatory and renoprotective effects of EOC that improve renal outcomes in experimental RAS.

RAS remains a major cause of renovascular hypertension and associated with progressive loss of renal mass and function.1 Furthermore, patients with RAS have substantially increased risks for cardiovascular disease, cerebrovascular disease, and mortality.2 Therefore, improved therapies to prevent cardiovascular and renal events in this disorder are urgently needed.

Renal revascularization by PTRA has become a mainstay for treatment of RAS, particularly with declining kidney function or refractory hypertension,10 yet fails to confer meaningful benefits for recovery of renal function beyond medical therapy alone.1,15 These observations are consistent with our previous data demonstrating that PTRA in swine RAS restores GFR and stenotic-kidney endothelial function, whereas renal perfusion, microvascular rarefaction, and interstitial fibrosis remain incompletely restored.4 We have also established the feasibility of cell-based therapy with EOC for preserving the stenotic-kidney microvascular architecture, hemodynamics, and function.2 However, EOC-alone do not reduce arterial pressure, hence important elements of target organ injury and cardiovascular risk are not reversed. The current study extends our previous observations and demonstrates that combining PTRA+EOC provides an opportunity to both decrease arterial pressure and recover kidney function.

EOC possess important renoprotective properties responsible for attenuating renal dysfunction and damage in chronic RAS.8,10 Augmentation of neovascularization in the injured kidney is mediated by engraftment and retention, and by paracrine secretion of angiogenic growth factors.16 In this study, a sizable fraction of injected cells was identified within the interstitium, renal tubules,9 or incorporated into CD31+ blood vessels, and some exhibited proliferation (proliferating cell nuclear antigen+/EOC). Furthermore, elevated VEGF immunoreactivity in EOC-treated pigs localized particularly in the vicinity of progenitor cells, linking them to local VEGF release. Taken together, these observations suggest sustained functionality (angiogenic and proliferating potential) of injected cells at harvest. Indeed, we have previously shown in swine RAS that EOC express and secrete VEGF into the culture.9
Importantly, increased renal expression of VEGF and VEGF receptor-2 in RAS+PTRA+EOC likely promoted vascular restoration, as well as endothelial nitric oxide synthase and basic fibroblast growth factor, angiogenic factors that promote vasodilation during the early angiogenesis, contributing to tubular epithelial repair. Moreover, we have previously shown in swine RAS that intrarenal delivery of EOC is associated with upregulation of EOC homing factors, such as stromal cell-derived factor-1 and its receptor C-X-C chemokine receptor type 4, as well as angiopoietin-1, an endothelial cell survival factor that promotes maturation of the new vessels and vascular repair. Upregulation of basic fibroblast growth factor and endothelial nitric oxide synthase expression observed after PTRA-alone suggest a role for reperfusion in regulating their expression. Nevertheless, microvascular density in RAS+PTRA kidneys remained diminished, possibly because of the unchanged VEGF expression and tubulointerstitial fibrosis.

The angiogenic potency of EOC might have contributed to the improved spatial density of cortical microvessels. Recent studies in swine RAS also showed increased microvascular density in stenotic-kidneys treated with VEGF. Selective loss of small microvessels observed in RAS and RAS+PTRA kidneys manifested in increased average vessel diameter that was restored in RAS+EOC and RAS+PTRA+EOC pigs. Similarly, EOC attenuated stenotic-kidney microvascular remodeling and vessel tortuosity, suggesting more mature vessel formation. Therefore, EOC induced both quantitative and qualitative improvement in microvascular architecture.

These changes of microvascular proliferation observed in EOC-treated pigs likely contributed to normalized levels of basal cortical perfusion and renal blood flow, which remained decreased in RAS+PTRA-alone–treated pigs, as previously shown. Interestingly, basal GFR and endothelial function were both restored to similar levels in RAS+EOC, RAS+PTRA, and RAS+PTRA+EOC pigs. Hence, both EOC and PTRA appear to independently improve renovascular responses to acetylcholine.

Several studies indicate that inflammation portends stenotic-kidney injury by increasing production and activity of cytokines, leading to microvascular damage, collagen deposition, matrix accumulation, and fibrosis. Interstitial macrophage accumulation in swine RAS stimulates fibrosis and vascular rarefaction. In the current study, stenotic-kidney release and systemic levels of MCP-1 declined to normal levels in RAS+EOC and RAS+PTRA+EOC pigs, likely decreasing macrophage abundance. Importantly, our results demonstrated that EOC decreased the number of M1 (CD68+/iNOS+) and increased the number of M2 (CD68+/Arg-1+) macrophages in vivo, implying a shift to a predominantly anti-inflammatory phenotype. Consistent with these observations, coculture of activated monocytes with EOC decreased expression of M1 markers (iNOS, TNFα, and nuclear factor kB), and increased M2 marker expression (IL-10 and Arg-1). To further elucidate mechanisms underlying the anti-inflammatory effects of EOC, we demonstrated that VEGF inhibition (but not IL-10 silencing) impeded the switch from M1 to M2 subpopulations. The contribution of VEGF was further confirmed by the observation that its intrarenal infusion in RAS animals decreased the number of M1 macrophages compared with RAS, normalizing M1/M2 ratio in vivo. These results uncovered a critical role of VEGF, underpinning the anti-inflammatory effects of EOC. Notably, intrarenal infusion of VEGF did not increase the number of M2 macrophages in vivo, and neither did its blockade prevent upregulation of IL-10 by EOC in vitro, implicating additional factors in the salutary effects of EOC on M2-macrophages.

Pertinently, EOC are known to express and secrete VEGF. Yet, to examine additional means of their communication with other cells, we isolated and characterized EOC-derived microvesicles. Small vesicles derived from the endosomal compartment can be internalized into endothelial cells and activate angiogenic pathways by delivering microRNA content. Recent data indicate that microvesicles derived from EOC carry VEGF and are able to trigger angiogenesis both in vitro and in vivo by a horizontal transfer of mRNA to endothelial cells leading to activation of phosphatidylinositol-3-kinase/protein kinase B signaling pathway and endothelial nitric oxide synthase. Indeed, we identified multiple microvesicles released from cultured EOC, which might serve as vehicles for EOC to signal a shift in renal macrophage phenotype. Additional studies are needed to pinpoint signals transmitted via these microvesicles and identify the cell types they target.

We believe that preferential differentiation toward an M2 subpopulation in EOC-treated kidneys may contribute to repair of the poststenotic kidney, and ameliorate renal and systemic inflammation by decreasing renal release of proinflammatory cytokines. Indeed, EOC-normalized stenotic-kidney inflammatory markers and the anti-inflammatory IL-10. We have previously demonstrated that the postischemic human kidney releases inflammatory cytokines that portend kidney injury and are linked to attenuated renal recovery after PTRA. M2 macrophages may also attenuate systemic inflammation by inhibiting proliferation of circulating lymphocytes. Overall, the combination of low release and systemic levels of inflammatory mediators with macrophage polarization toward a trophic phenotype could have contributed to the attenuation of microvascular remodeling and fibrosis in animals treated with EOC.

Intrarenal infusion of EOC per se also attenuated apoptosis, reflected by reduced number of TUNEL+ and caspase-3+ cells. Furthermore, concurrent PTRA+EOC decreased the expression of Bax and normalized Bax/Bcl-xl ratio, implying a potentiated antiapoptotic effect. Notably, PTRA-alone decreased TUNEL positivity, but not caspase-3 immunoreactivity or Bax/Bcl-xl ratio, implying residual apoptotic activity. These results are consistent with the observation that paracrine factors released from EOC specifically decrease endothelial cell apoptosis by induction of prosurvival signals. In addition, in a mouse model of ureteral obstruction, M1-suppression decreased cellular apoptosis. Therefore, our results support an antiapoptotic effect of EOC, possibly secondary to transition from proapoptotic (M1) to antiapoptotic (M2) macrophage phenotypes.

Increased oxidative stress is responsible for many deleterious processes in the stenotic kidney, such as endothelial...
dysfunction and microvascular rarefaction, amplifying renal dysfunction and injury. In experimental atherosclerotic RAS, intrarenal delivery of EOC blunted its functional deterioration by decreasing renal oxidative stress. In agreement, our observations demonstrate that EOC effectively attenuated in situ production of superoxide anion, and PTRA+EOC particularly downregulated nitrotyrosine expression.

Tubulointerstitial fibrosis was also attenuated in animals treated with PTRA+EOC, likely secondary to downregulation of TGF-β and plasminogen activator inhibitor-1. We recently observed elevated tissue TGF-β immunoreactivity in hyperperfused compared with healthy human kidneys, which correlated with higher number of CD68+ macrophages, suggesting a link between TGF-β and inflammatory cell infiltration. Similarly, in swine atherosclerotic RAS, persistent renal fibrosis, secondary to upregulation of TGF-β and plasminogen activator inhibitor-1, was associated with impaired microvascular architecture and renal dysfunction after revascularization. Therefore, downregulation of these markers may have attenuated the inflammatory response and permitted microvascular proliferation in EOC-treated pigs. Congruently, fibronectin and collagen IV content were attenuated after EOC infusion, which might have decreased renal injury and promoted renal recovery potential.

In contrast to RAS+EOC and RAS+PTRA that partly restored renal structural and functional parameters, only concurrent EOC and PTRA fully normalized serum creatinine levels. Similarly, only this combination upregulated VEGF expression compared with RAS. Therefore, the combination of superior angiogenic potential, a decrease in blood pressure, and improved renal function, translated into better renal outcomes after revascularization.

Limitations

Our study is limited by the use of relatively young animals and the absence of comorbid conditions, such as essential hypertension or diabetes mellitus, common in human RAS. Nevertheless, renal structure and function are similar to human kidneys in our swine model, which recapitulates many characteristics of human renovascular hypertension. The optimal timing and doses of EOC, and their long-term benefits, will need to be determined in future studies. Additional studies will also need to explore other effects of EOC and their underlying mechanisms.

Conclusions

The current study shows that EOC evokes a shift likely VEGF-mediated in macrophage phenotype from a proinflammatory type to a population more permissive for renal repair and regeneration. A single intrarenal infusion of EOC during revascularization preserved the microvascular network and function, and ameliorated inflammation, oxidative stress, and renal scarring beyond the stenotic lesion. Moreover, serum creatinine levels were normalized. Therefore, our study suggests a novel therapeutic role for adjunctive EOC delivery in decreasing inflammation, promoting renal repair, and thereby improving renal recovery potential in chronic experimental RAS. Further research is needed to examine the persistence of the beneficial effects of this approach in human renovascular disease.

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Disclosures

None.

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**Significance**

Renal revascularization represents an important treatment modality for certain selected patients with renal artery stenosis, but clinical studies failed to demonstrate meaningful benefits for recovery of renal function beyond medical therapy alone, warranting the need for mandating development of effective adjunctive therapies. Endothelial outgrowth cells (EOC) possess unique capacity to repair ischemic tissues, representing a novel treatment option to salvage the stenotic-kidney. This study demonstrates for the first time that a single intrarenal infusion of EOC at the time of revascularization attenuated tissue injury and improved renal outcomes by inducing a shift of renal macrophage subpopulations from an inflammatory to reparative phenotype. The mechanism for this effect appears to be partly vascular endothelial growth factor (VEGF)-dependent. These results reveal immunomodulatory and renoprotective effects of adjunctive EOC delivery in conjunction with revascularization to improve renal outcomes in experimental renovascular disease.
Endothelial Outgrowth Cells Shift Macrophage Phenotype and Improve Kidney Viability in Swine Renal Artery Stenosis


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**Supplemental Material**

*Table I.* Efficacy of different therapeutic strategies to improve renal function and structure in renal artery stenosis (RAS) 4 weeks after treatment in comparison to untreated RAS.

<table>
<thead>
<tr>
<th></th>
<th>EOC</th>
<th>PTRA</th>
<th>PTRA+EOC</th>
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<tbody>
<tr>
<td><strong>Stenotic kidney</strong></td>
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<td></td>
<td></td>
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<tr>
<td>Blood supply</td>
<td>↑↑</td>
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<tr>
<td>GFR</td>
<td>↑↑</td>
<td>↑↑</td>
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<tr>
<td>Endothelial function</td>
<td>↑↑</td>
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<tr>
<td>Neovascularization</td>
<td>↑</td>
<td>↑</td>
<td>↑↑</td>
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<tr>
<td>M1/M2</td>
<td>↓↓</td>
<td>↔</td>
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</tr>
<tr>
<td>Glomerulosclerosis</td>
<td>↓</td>
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<tr>
<td>Tubulo-interstitial fibrosis</td>
<td>↓</td>
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<tr>
<td><strong>Systemic</strong></td>
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<tr>
<td>Mean arterial pressure</td>
<td>↔</td>
<td>↓↓</td>
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<tr>
<td>Serum creatinine</td>
<td>↔</td>
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<tr>
<td>Inflammation</td>
<td>↓</td>
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</tbody>
</table>

↑↑: p>0.05 vs. normal, ↑: p<0.05 vs. RAS but p<0.05 vs. normal, ↔: p<0.05 vs. normal and p>0.05 vs. RAS.
Timeline describing sequence of events in experimental RAS+EOC (A), RAS+PTRA (B), and RAS+PTRA+EOC (C) and comparing the evolution of mean arterial pressure (MAP) measured by telemetry, renal angiogram after PTRA or Sham, and serum creatinine levels. *p<0.05 vs. normal.
Figure III

A: Mean arterial pressure (telemetry) decreased after PTRA. B: Representative fluorescence of CM-Dil (red, 20x) EOC and cytokeratin (green) stained in the stenotic kidney 4 weeks after administration. Blue: DAPI-stained nuclei. Injected EOC were detected at the interstitium (yellow arrows) and renal tubules (white arrows).
Representative fluorescence of CM-Dil (red, 40x) EOC and PCNA (A, green) or CD31 (B, green) stained in the stenotic kidney 4 weeks after administration. Blue: DAPI-stained nuclei.
A: Representative immunofluorescence staining for CD34 (red) and VEGF (green) in the stenotic kidney 4 weeks after PTRA+EOC. B: Quantification of VEGF immunoreactivity. Blue: DAPI-stained nuclei. *p<0.05 vs. EOC-treated.
Representative immunofluorescence staining with PCNA (top) and its quantification (bottom). *p<0.05 vs. normal, †p<0.05 vs. RAS+PTRA+EOC, ‡p<0.05 vs. RAS.
Representative TUNEL (green) and caspase-3 (red) staining (A) showing decreased number of apoptotic cells (arrows) in EOC-treated pigs. B: Renal production of superoxide anion, detected by dihydroethidium (40x).
A: Representative immunofluorescence (40x) for M1 (anti-macrophage CD68/ inducible nitric-oxide synthase (iNOS)), and M2 CD68/arginase (Arg)-1 macrophages. B: Protein expression of iNOS, TNF-α, nuclear factor (NF)κB, interleukin (IL)-10, and Arg-1 in cultured activated monocytes with or without untreated endothelial progenitor cells (EOC), or EOC pretreated with vascular endothelial growth-factor (VEGF) antibody, or IL-10 silencing. §p<0.05 vs. monocytes, ||p<0.05 vs. monocytes+EOC.
A: Representative immunoblots showing contiguous bands for PAI-1, VEGFR-2, VEGF, eNOS, TGF-β, TIMP-2, bFGF, nitrotyrosine, p47, Bax, Bcl-xl, and GAPDH. Stenotic-kidney net release of interferon (IF)-γ (B), tumor necrosis factor (TNF)-α (C), monocyte-chemoattractant-protein (MCP)-1, and interleukin (IL)-10 in NORMAL, NORMAL+EOC, RAS, RAS+EOC, RAS+PTRA, and RAS+PTRA+EOC pigs. *p<0.05 vs. normal, #p<0.05 vs. RAS+PTRA+EOC, ‡p<0.05 vs. RAS.
Representative immunostaining (40x) of fibronectin and collagen IV (A). D: Representative renal trichrome staining (40x) in the experimental groups (B).
Transmission electron microscopy showing release of microvesicles from cultured EOC (A x3900) (B x26500). (C) Characterization of EOC-derived microvesicles by their expression of α4 integrin, β1 integrin, CD34, HLA class I, CD42b, p selectin, and CD14 detected by fluorescence activated cell sorting (FACS) analysis.
Representative immunofluorescence staining (40x) for M1 (anti-macrophage CD68 / inducible nitric oxide synthase (iNOS)), and M2 CD68/arginase (Arg)-1 macrophages and their quantification (B). C: iNOS/Arg-1 ratio. *p<0.05 vs. normal, †p<0.05 vs. RAS+VEGF.