Selective Stimulation of VEGFR2 Accelerates Progressive Renal Disease

Vascular endothelial growth factor A (VEGF-A) can play both beneficial and deleterious roles in renal diseases, where its specific function might be determined by nitric oxide availability. The complexity of VEGF-A in renal disease could in part be accounted for by the distinct roles of its two receptors; VEGFR1 is involved in the inflammatory responses, whereas VEGFR2 predominantly mediates angiogenesis. Because nondiabetic chronic renal disease is associated with capillary loss, we hypothesized that selective stimulation of VEGFR2 could be beneficial in this setting. However, VEGFR2 activation may be deleterious in the presence of nitric oxide deficiency. We systematically overexpressed a mutant form of VEGF-A binding only VEGFR2 (Flk-sel) using an adeno-associated virus-1 vector in wild-type and eNOS knockout mice and then induced renal injury by uninephrectomy. Flk-sel treatment increased angiogenesis and lowered blood pressure in both mouse types. Flk-sel overexpression caused mesangial injury with increased proliferation associated with elevated expression of PDGF, PDGF-β receptor, and VEGFR2; this effect was greater in eNOS knockout than in wild-type mice. Flk-sel also induced tubulointerstitial injury, with some tubular epithelial cells expressing α-smooth muscle actin, indicating a phenotypic evolution toward myofibroblasts. In conclusion, prestimulation of VEGFR2 can potentiate subsequent renal injury in mice, an effect enhanced in the setting of nitric oxide deficiency. (Am J Pathol 2011, 179: 155–166; DOI: 10.1016/j.ajpath.2011.03.024)

Vascular endothelial growth factor A (VEGF-A) plays a key role in maintaining peritubular and glomerular capillary integrity in the normal kidney. In renal disease, however, the actions of VEGF-A are more complicated. A reduction in renal VEGF-A is observed in acute and chronic nondiabetic renal disease, mostly associated with a loss of glomerular and peritubular capillaries. In these situations, administration of VEGF-A has been shown to improve renal histology and function. In contrast, levels of both circulating and local VEGF-A are high in diabetes, and excessive VEGF-A has been shown to have a role in mediating glomerular hypertrophy, proteinuria, and retinopathy, indicating that VEGF-A is deleterious in this unique situation. Given the fact that diabetic conditions are associated with a lower bioavailability of nitric oxide (NO), we have proposed a hypothesis that the deleterious effect of VEGF-A in diabetes could be attributed to a reduced bioavailability of endothelial NO in the kidney. Consistent with this hypothesis, diabetic nephropathy is worsened in the setting of endothelial NO deficiency and the effects of VEGF-A to induce angiogenesis and inflammation are enhanced in this setting.

The actions of VEGF-A are mediated through its two receptors, Flt-1 (VEGFR1) and Flik-1 (VEGFR2). VEGFR1 is involved in the inflammatory response by stimulating macrophage chemotaxis, as well as vascular permeability and vessel stabilization. In contrast, VEGFR2 mediates angiogenesis and reduces blood pressure. The specific roles of VEGFR1 and VEGFR2 in nondiabetic and diabetic kidney disease are not well understood.

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Address reprint requests to Takahiko Nakagawa, M.D., Ph.D., Division of Renal Disease and Hypertension, University of Colorado Denver, C281, Aurora, CO 80045. E-mail: Takahiko.Nakagawa@ucdenver.edu.
One could postulate that VEGFR2 (but not VEGFR1) might have a key role in protecting the kidney from non-diabetic injury by preserving capillary number, because of its angiogenic effects. In diabetic nephropathy, however, VEGFR2 likely has a causal role, as is suggested by studies showing up-regulation of VEGFR2 mRNA expression within weeks of the onset of diabetes in the rat, whereas VEGFR1 was undetectable. In this situation, a reduction in endothelial NO, which is often observed in diabetic patients, might be a key factor to enhance VEGFR2 signaling for excessive angiogenesis.

To begin to understand the roles of VEGFR1 and VEGFR2 in diabetic and nondiabetic kidney diseases, we generated an adeno-associated virus containing a mutant form of VEGF-A that binds only to VEGFR2 (rAAV1-Flk-sel) and injected these vectors in normal wild-type (WT) mice and in eNOS-knockout mice (eNOSKO) lacking endothelial NO synthase. Because the role of a specific factor can be more evident in the presence of renal injury than in normal kidney, uninephrectomy (UNx) was performed to accelerate renal injury. We hypothesized that administration of rAAV1-Flk-sel in UNx-WT mice would be protective by enhancing angiogenesis of glomerular and peritubular capillaries. In contrast, we predicted that overexpression of rAAV1-Flk-sel in UNx-eNOSKO mice would be deleterious. Overexpression of the VEGF-A mutant for VEGFR1 was unsuccessful in our hands; therefore, here we present the results only for overexpression of Flk-sel.

Materials and Methods

Experimental Protocol

Eight-week-old male C57BL/6J-Nos3tm1Unc mice (eNOSKO mice) and background strain C57BL6/J mice (WT mice) weighing 20 to 25 g were purchased from the Jackson Laboratory (Bar Harbor, ME). Mice were housed under a 12-hour light/dark cycle with free access to food and water. All experiments were performed in accordance with the Animal Care and Use Committee of the University of Florida. Mice (n = 10 in each group) were injected intramuscularly with 1.0 × 10^9 viral particles of either rAAV1-empty vector (EV) or rAAV1-Flk-sel. This level was chosen because a lower dose of 1.0 × 10^9 viral particles failed to increase serum VEGF-A levels in pilot studies. Flk-sel was provided by Dr. Napoleon Ferrara (Genentech, South San Francisco, CA). The construction and characterization of Flk-sel has been described previously, with mutations in human VEGF_165 at positions D63S/G65M/L66R. The rAAV vectors expressing Flk-sel were generated and purified in the Vector Core Laboratory at the University of Florida, using techniques described previously. Three months later, UNx was used to induce kidney injury. Renal histology was examined at 4 months with blood and urine collected to measure blood urea nitrogen and urinary albumin/creatinine ratio, as described previously. The scheme of the experimental design is presented in Figure 1.

Blood Pressure Measurements

Systolic blood pressure (BP) was assessed every month using a tail-cuff sphygmomanometer (Visitech BP2000; Visitech Systems, Apex, NC). Animals were accustomed to the machine by training, and all measurements were performed at the same time of day.

Serum VEGF-A Measurement

Serum VEGF-A levels were measured at 3 and 4 months using a commercial human enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions. We had previously confirmed that Flk-sel can be detected by this kit.

IHC

Kidneys were fixed in neutral buffered formalin (10%), embedded in paraffin, and sectioned at 2-μm thickness. Tubulointerstitial injury was assessed by immunohistochemistry (IHC) with goat anti-human collagen III (SouthernBiotech, Birmingham, AL) and rat anti-mouse F4/80 antibody (Serotec, Oxford, UK) which detects a specific phenotype of macrophages present predominantly in the interstitium. Phenotypic changes in tubular epithelial cells were evaluated using rabbit anti-human α-SMA (Abcam, Cambridge, MA) and rabbit anti-human transforming growth factor β1 (TGF-β1) (Santa Cruz Biotechnology, Santa Cruz, CA). Rabbit anti-mouse collagen IV (Chemicon International, Temecula, CA), rabbit anti-human fibronectin (Sigma-Aldrich, St. Louis, MO), and mouse anti-human CD68 (Abcam; performed on frozen kidney), a marker of macrophage subtypes predominantly expressed in glomeruli, were used to evaluate glomerular injury. For vascular assessment of angiogenic responses, capillary endothelial cells were detected by a rabbit anti-rat thrombomodulin (Santa Cruz Biotechnology, Santa Cruz, CA) and rabbit anti-human platelet-derived growth factor receptor-β (PDGFR-β) (Santa Cruz Biotechnology), rabbit anti-human PDGF-B (Ab-1; Oncogene Research Products, La Jolla, CA), or anti-human PDGF-D (kindly provided by ZymoGenetics, Seattle, WA) to examine mesangial damage, followed by ImmPress anti-rabbit Ig polymer detection system (Vector Laboratories, Burlingame CA). To examine cell proliferation, the thymidine analog 5-bromo-2′-deoxynucleoside (BrdU) was used to induce kidney injury. Mice were killed by cervical dislocation and kidneys were processed for IHC.

Figure 1. Study protocol. Three months after adeno-associated virus (AAV) injection, UNx was used to induce kidney injury. Mice were sacrificed at 4 months.
oxyuridine (BrdU) (50 mg/kg; Sigma-Aldrich) was intra-peritoneally administered to mice 2 hours before sacrifice. BrdU-positive cells were detected with a rat monoclonal BrdU antibody (Serotec). For all antibodies, color was developed using 3,3′-diaminobenzidine (Dako, Carpinteria, CA). For negative controls, primary antibodies were replaced with species-matched antibodies.

Morphological Quantification

Periodic acid-Schiff was used to detect morphological characteristics. All glomeruli (>50 glomerular cross-sections per biopsy) and the whole cortical tubulointerstitial area in the axial plane were scanned with the AxioVision image analyzer (Carl Zeiss, Thornwood, NY) and examined. The total dimension of the glomerulus was determined by tracing the

Table 1. General Characteristics of Mice at 3 Months after rAAV Injection

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>EV</th>
<th>Flik-sel</th>
<th>EV</th>
<th>Flik-sel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>28.0 ± 2.0</td>
<td>30.0 ± 2.3*</td>
<td>27.3 ± 1.7</td>
<td>31.1 ± 3.3†</td>
</tr>
<tr>
<td>Kidney weight (g)</td>
<td>0.16 ± 0.014</td>
<td>0.16 ± 0.021</td>
<td>0.14 ± 0.011</td>
<td>0.15 ± 0.011</td>
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<tr>
<td>K/B (10⁻²)</td>
<td>0.57 ± 0.05</td>
<td>0.55 ± 0.08</td>
<td>0.52 ± 0.05</td>
<td>0.49 ± 0.06</td>
</tr>
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</table>

*P < 0.05 versus WT-EV.
†P < 0.01 versus eNOSKO-EV.
eNOSKO, eNOS knockout; EV, empty vector; Flik-sel, VEGFR2-selective; rAAV, recombinant adeno-associated virus.
Western Blot Analysis

Mouse kidney tissues were snap-frozen in liquid nitrogen for protein isolation. Western blot analysis was performed with 10 to 30 μg protein/lane. Blots were incubated with either rabbit monoclonal VEGFR1 antibody (Epitomics, Burlingame, CA), rabbit monoclonal VEGFR2 antibody (Cell Signaling), mouse monoclonal β-actin antibody (Sigma-Aldrich), rabbit anti-mouse Akt (both total Akt and phosphorylated Ser473) (Cell Signaling), mouse monoclonal anti-human GAPDH (Santa Cruz Biotechnology), rabbit anti-TGF-β1 (Cell Signaling), rabbit anti-HIF1α (Novus Biologicals, Littleton, CO), a rabbit anti-human α-SMA antibody (Abcam), or a mouse anti-human E-cadherin antibody (BD Transduction Laboratories, San Diego, CA), followed by incubation with peroxidase-conjugated rabbit IgG or mouse IgG (DakoCytomation, Carpinteria, CA). Receptor phosphorylation of VEGFR1 was detected using a rabbit anti-phospho-Flt-1 (pY1213) antibody (Millipore-Chemicon International, Temecula, CA). Phosphorylated VEGFR2 was detected by a rabbit anti-mouse-pY945-VEGFR R2 antibody (BioSource, Camarillo, CA). Proteins were visualized with an enhanced chemiluminescence detection system (Amer sham-GE Healthcare, Piscataway NJ). The density of each band was measured using public domain ImageJ software version 1.41 (NIH, Bethesda, MD).

Electron Microscopy

Kidneys were fixed in glutaraldehyde, postfixed with osmium tetroxide, and embedded in Epon 812 medium (Nisshin EM, Tokyo, Japan). Ultrathin sections were examined with a H7100 electron microscope (Hitachi, Ibaraki, Japan).

Table 2: General Characteristics of Mice at 1 Month after Uninephrectomy

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Wild type</th>
<th>Fik-sel</th>
<th>eNOSKO</th>
<th>Fik-sel</th>
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</thead>
<tbody>
<tr>
<td>Body weight B (g)</td>
<td>27.2 ± 1.8</td>
<td>31.6 ± 3.2***</td>
<td>26.9 ± 2.0</td>
<td>30.6 ± 5.8‡</td>
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<tr>
<td>Kidney weight K (g)</td>
<td>0.20 ± 0.014</td>
<td>0.20 ± 0.0088</td>
<td>0.17 ± 0.020</td>
<td>0.19 ± 0.012†</td>
</tr>
<tr>
<td>K/B (10⁻⁵)</td>
<td>0.75 ± 0.05</td>
<td>0.65 ± 0.05**</td>
<td>0.62 ± 0.06**</td>
<td>0.55 ± 0.09</td>
</tr>
<tr>
<td>BUN (mg/dL)</td>
<td>28.7 ± 3.4</td>
<td>34.2 ± 3.9*</td>
<td>37.8 ± 2.1****</td>
<td>41.9 ± 3.8†</td>
</tr>
<tr>
<td>u-Alb/u-Cre</td>
<td>0.12 ± 0.07</td>
<td>0.48 ± 0.07****</td>
<td>0.88 ± 0.39***</td>
<td>0.70 ± 0.50</td>
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*p < 0.05, **p < 0.01, ***p < 0.005, and ****p < 0.001 versus WT-EV.
†p < 0.05 and ‡p < 0.001 versus eNOSKO-EV.
BUN, blood urea nitrogen; eNOSKO, eNOS knockout; EV, empty vector; Fik-sel, VEGFR2-selective; u-Alb/u-Cre, urine-albumin/urine creatinine ratio; WT, wild type.

Cell Culture

Human renal proximal tubular (HK2) cells were cultured in keratinocyte-SFM medium containing epidermal growth factor, bovine pituitary extracts, and 10% serum (Invitrogen, Carlsbad, CA). Ten percent serum was added in order to keep cells healthy during stimulation for 72 hours. After

Figure 3. Time course of systolic blood pressure. The rAAV1-Flk-sel treatment lowered blood pressure (BP) beginning at 2 months after injection in WT mice and the change was sustained in UNx-WT mice. BP was significantly higher in eNOSKO mice, compared with WT mice before treatment, but administration of rAAV1-Flk-sel led to a significant decrease 1 month later. The lowering of BP by rAAV1-Flk-sel was also observed in UNx-eNOSKO mice. Solid symbols, eNOSKO mice; open symbols, WT mice. Squares, rAAV1-EV; circles, rAAV1-flk-sel. *p < 0.001 versus WT mice treated with rAAV1-EV; †p < 0.01 versus eNOSKO mice treated with rAAV1-EV; ‡p < 0.001 versus eNOSKO mice treated with rAAV1-EV; §p < 0.05 versus WT mice treated with rAAV1-EV; §p < 0.01 versus WT mice treated with rAAV1-EV. Data are expressed as means ± SD, n = 10 in each group.
subconfluence, cells were stimulated by recombinant human VEGF-A 165 (100 ng/mL; PeproTech, Rocky Hill, NJ) in the presence of 10% serum to examine if VEGF alters the expressions of E-cadherin and α-smooth muscle actin (α-SMA), analyzed by Western blotting at 24, 48 and 72 hours as described above.

**Statistical Analysis**

All values are expressed as means ± SD. Statistical analysis was performed with unpaired, two-tailed Student’s *t*-tests for single comparisons or analysis of variance with post hoc testing using Tukey’s method for multiple comparisons. A *P* value of <0.05 was taken to indicate a significant difference.

**Results**

**Systemic Overexpression of rAAV1-Flk-sel in WT and eNOSKO Mice**

Initial experiments were performed to determine whether rAAV1-Flk-sel had any effect on WT and eNOSKO mice without renal disease. We administered rAAV1-Flk-sel intramuscularly to both WT and eNOSKO mice and examined them 3 months later. Injection of rAAV1-Flk-sel led to significant elevation of systemic human VEGF-A at 3 months after injection in both mouse types (*P* < 0.01 versus rAAV1-EV in WT and eNOSKO mice) (Figure 2A), and levels were similar in both mouse types. eNOSKO mice exhibited mild mesangial expansion (Figure 2D), compared with WT mice (Figure 2B), probably because of eNOS deficiency at this age.28 rAAV1-Flk-sel did not alter renal morphology (Figure 2, C and E) or kidney weight in either mouse type (Table 1). Unfortunately, neither blood urea nitrogen nor urine albumin excretion was examined at this point. No remarkable morphological abnormalities were found in spleen, heart, or liver in the mice at this point (data not shown).

Given these findings, we then induced renal injury by performing UNx in both WT and eNOSKO mice at 3 months after rAAV1-Flk-sel injection and examined the mice 1 month later. Both UNx-WT and UNx-eNOSKO mice treated with rAAV1-Flk-sel exhibited an increase in renal total and phosphorylated VEGFR2 (Figure 2, F–H). We did not observe any changes in VEGFR1 levels, and there was no evidence of VEGFR1 phosphorylation in any of the groups serve any changes in VEGFR1 expression. We also performed UNx in both WT and eNOSKO mice (Figure 2, J and K). These
data suggest that renal VEGF-A was successfully stimulated by rAAV1-Flk-sel administration in this model.

rAAV1-Flk-sel overexpression was associated with increased body weight in both UNx-WT and UNx-eNOSKO mice, compared with rAAV1-EV treatment (Figure 2L and Table 2). Although previous studies indicated that VEGF-A causes renal hypertrophy,30,31 rAAV1-Flk-sel treatment did not alter kidney size after UNx; instead, the ratio of kidney to body weight was reduced (Table 2). Although overexpression of rAAV1-Flk-sel was well tolerated by both WT and eNOSKO animals, it did lead to some changes in renal function after UNx (Table 2). UNx-WT mice treated with rAAV1-Flk-sel had elevated blood urea nitrogen and urinary albumin excretion, compared with those treated with rAAV1-EV. Blood urea nitrogen was higher in UNx-eNOSKO than in UNx-WT animals, and rAAV1-Flk-sel treatment further enhanced levels. rAAV1-Flk-sel administration to UNx-eNOSKO mice did not lead to alterations in albumin excretion.

Role of VEGFR2 in Blood Pressure in WT and eNOSKO Mice

Consistent with reports that VEGFR2 is responsible for reducing BP,16 we found rAAV1-Flk-sel WT mice had lower systemic BP than rAAV1-EV WT mice (Figure 3), both before UNx and at 1 month after. Given that the effect of VEGF-A on lowering BP is thought to be secondary to stimulating endothelial NO,32 we had hypothesized that BP would not be lowered in the eNOSKO mouse. However, BP was unexpectedly lowered in eNOSKO mice, with rAAV1-Flk-sel overexpression before and at 1 month after UNx (Figure 3).

Increased Angiogenesis in the Kidneys of Flk-sel Overexpressing Mice

One of the key actions of VEGF-A is to enhance endothelial cell proliferation. We therefore examined BrdU incorporation, as a marker of cell proliferation,33 and thrombomodulin, as a specific endothelial cell marker24,34 (Figure 4). Administration of rAAV1-Flk-sel tended to increase thrombomodulin-positive endothelial cells in glomeruli and tubulointerstitium in UNx-WT mice. This effect was significantly pronounced in the UNx-eNOSKO mice (Figure 4, G and J). The increase in endothelial cells was associated with induction of proliferating cells labeled with BrdU (Figure 4, H and K). Double staining of thrombomodulin with BrdU confirmed an increase in proliferating endothelial number by rAAV1-Flk-sel, which was enhanced in UNx-eNOSKO animals (Figure 4I). Counts of glomerular capillaries were also elevated by rAAV1-Flk-sel in both
UNx-WT and UNx-eNOSKO mice, but a lack of eNOS had no effect on this parameter (Figure 5H).

De Novo Mesangial Proliferative Glomerular Injury

We had postulated that overexpression of rAAV1-Flk-sel would be beneficial in UNx-WT mice, because of its ability to lower BP and stimulate angiogenesis; however, unexpectedly, a marked mesangial expansion and proliferation were induced. rAAV1-Flk-sel induced mesangial expansion, glomerular hypertrophy, and mesangial proliferation in UNx-WT and UNx-eNOSKO mice (Figure 5, A–G), but there was no alteration in mesangiolysis (data not shown). Enhanced mesangial expansion was accompanied by elevation in collagen IV and fibronectin deposition (Figure 5, I–N). An increase in CD68$^+$ glomerular macrophages after rAAV1-Flk-sel treatment in both UNx-WT and UNx-eNOSKO mice also likely contributed to the high cellularity in the mesangium. However, a lack of eNOS had no effect on this response (Figure 5O).

Electron microscopy confirmed these findings, with a marked increase of mesangial cells (Figure 6A) and subendothelial vacuolization (Figure 6B) in UNx-eNOSKO mice overexpressing rAAV1-Flk-sel. Narrowing of the capillary lumen was concomitant with endothelial swelling and mesangial cell proliferation and interposition in UNx-eNOSKO mice treated with rAAV1-Flk-sel (Figure 6C). Podocyte cell bodies had a normal appearance, whereas focal foot process fusion was occasionally observed in UNx-eNOSKO mice, with or without treatment (Figure 6, D and E).

The PDGF-B and -D isoforms are key cytokines in regulating mesangial cell proliferation. We found that PDGF-D was barely expressed in glomeruli of UNx-WT mice but was significantly induced by rAAV1-Flk-sel treatment (Figure 7, A and B). A lack of eNOS itself also induced mesangial PDGF-D expression, compared with nontreated UNx-WT mice, and this was greater in the presence of rAAV1-Flk-sel overexpression (Figure 7, C and D). PDGF-B and PDGF-βR expression mirrored the PDGF-D staining patterns in these mice (Figure 7, E–L). The observation that overexpression of rAAV1-Flk-sel resulted in mesangial expansion and proliferation led us to closely evaluate the sites of expression of VEGFR2 in this model. Although it is predominantly expressed in endothelial cells, VEGFR2 has been reported in mesangial cells of diseased glomeruli, and VEGFR2 was found to be induced in the mesangial cells of UNx-WT mice after rAAV1-Flk-

![Figure 6. Glomerular ultrastructure in UNx-eNOSKO mice treated with rAAV1-Flk-sel. Representative electron micrographs demonstrate increased mesangial cell (Mc) number (A) and extracellular matrix deposition (A and B) in glomeruli of UNx-eNOSKO mice treated with rAAV1-Flk-sel. B: Vacuolization is observed in the subendothelial space (arrows). C: A narrowing of glomerular capillary lumen (CL) (arrows) due to swelling of the endothelial cell (Ec) with partial mesangial interposition is present in UNx-eNOSKO mice treated with rAAV1-Flk-sel treatment. In contrast, podocytes (Pd) appear normal (D), although some foot processes are fused (arrows) (E).](image)

![Figure 7. Glomerular expression of PDGF family and VEGFR2. A–P: Staining (brown) for PDGF-D, PDGF-B, PDGF-βR, and VEGFR2 in glomeruli of four groups of mice 1 month after UNx: UNx-WT mice treated with rAAV1-EV (A, E, I, and M) or with rAAV1-Flk-sel (B, F, J, and N); UNx-eNOSKO mice treated with rAAV1-EV (C, G, K, and O) or with rAAV1-Flk-sel (D, H, L, and P). Scale bars = 20 μm. Q: Quantitative analysis for % positive VEGFR2 expression in glomerulus (glomer). *P < 0.01, ***P < 0.001. Data are expressed as means ± SD; n = 10 in each group.](image)
Flk-sel treatment (Figure 7, M, N, and O). Furthermore, VEGFR2 was also expressed in UNx-eNOSKO mice and was greatly enhanced with rAAV1-Flk-sel treatment. Collagen III deposition was more severe in UNx-eNOSKO mice treated with rAAV1-EV than in UNx-WT mice treated with rAAV1-EV, whereas rAAV1-Flk-sel treatment significantly induced interstitial collagen III deposition in UNx-eNOSKO mice (Figure 8, C–E). F4/80-positive macrophage infiltration was induced by rAAV1-Flk-sel treatment in both eNOSKO and WT mice (Figure 8F).

**Tubulointerstitial Injury**

Mild tubulointerstitial injury was observed in UNx-eNOSKO mice treated with rAAV1-EV, as evidenced by tubular dilation (Figure 8, A and B) and atrophy accompanied by detachment of tubular epithelial cells (data not shown); the injury was more prominent in UNx-eNOSKO mice treated with rAAV1-Flk-sel. Collagen III deposition was more severe in UNx-eNOSKO mice treated with rAAV1-EV than in UNx-WT mice treated with rAAV1-EV, whereas rAAV1-Flk-sel treatment significantly induced interstitial collagen III deposition in UNx-eNOSKO mice (Figure 8, C–E). F4/80-positive macrophage infiltration was induced by rAAV1-Flk-sel treatment in both eNOSKO and WT mice (Figure 8F).

Expression of TGF-β1 (Figure 8, G and H), PDGF-B (Figure 8, J, K, and O), and VEGFR2 (Figure 8, L, M, and P) was increased in damaged tubular epithelial cells in UNx-eNOSKO and was enhanced with rAAV1-Flk-sel treatment. In particular, TGF-β1 expression was located in apical membrane of damaged tubular cells (Figure 8H); total protein levels of TGF-β1 were increased in whole kidney lysates of UNx-eNOSKO animals and were further enhanced by Flk-sel stimulation (Figure 8, I and N).

Strikingly, the damaged tubular epithelial cells expressed α-SMA, a marker of myofibroblast in UNx-eNOSKO mice, which was further enhanced by rAAV1-Flk-sel treatment (Figure 9, A and B). Consistently, total α-SMA expression in the kidney as indicated by Western blotting was elevated in these groups (Figure 9, C and D). To determine whether VEGF directly induces α-SMA on tubular epithelial cells, we conducted an in vitro study with HK2 cells. Consistent with our in vivo data, we found that VEGF-A induced α-SMA at 48 and 72 hours. In addition, VEGF-A also reduced E-cadherin expression, an epithelial cell marker (Figure 9E).
A proposed mechanism accounting for effects of specific VEGFR-2 stimulation is presented in Figure 10.

Discussion

In the present study, the primary finding was that supra-physiological activation of VEGFR2 before UNx predisposed WT mouse kidneys to renal injury. This finding was surprising, because VEGF-A administration has been reported to be beneficial in several types of renal injury.3,4,6,7 A potential explanation for this discrepancy is the timing of VEGF-A treatment and whether this maneuver occurs before or after onset of renal injury. The progression of renal injury is usually associated with a loss of capillaries, and so preventing capillary loss by VEGF-A might be beneficial. In contrast, the present study examined the effect of VEGF-A therapy performed before the onset of renal injury. Here, VEGF-A overexpression has the potential to lead to excessive numbers of vessels, which could be structurally and functionally immature38 and the presence of which may augment renal disease progression.

We found that stimulation of VEGFR2 caused abnormal angiogenesis in the kidney, and VEGF-A also contributed to the development of other renal injury features, including mesangial expansion, macrophage influx, and tubulointerstitial injury, in particular in the presence of NO deficiency. Of note, VEGF-A acting through the activation of VEGFR2, but not VEGFR1, is known to play a key role in diabetic nephropathy,17 which is characterized by phenotypes similar to those observed in the present study. Others have shown that VEGF-A inhibition prevents an increase in glomerular volume in diabetic animals.8,9,30 It is therefore possible that high VEGF-A, in addition to high glucose, could potentially contribute not only to the development of angiogenesis but to other features of diabetic glomerular injury. In this regard, giving Fk-sel therapy to diabetic patients might lead to further deterioration in diabetic nephropathy.

There are several potential mechanisms for how VEGF-A can alter mesangial cell biology. VEGF-A may directly bind to VEGFR2 expressed on mesangial cells. Previous studies have shown that VEGFR2 is exclusively expressed in endothelial cells in the normal mouse glomerulus,36 but we found VEGFR2 expression in the mesangial area of UNx-WT mice after Fk-sel treatment. Furthermore, eNOS deficiency also induced VEGFR2 expression, which was enhanced with Fk-sel treatment in UNx-eNOSKO mice. Given that Thomas et al37 also demonstrated VEGFR2 expression in mesangium in human proliferative glomerulonephritis, these cells may express VEGFR2 under certain conditions. The precise mechanism for elevated VEGFR2 in this model is unclear, but one possibility is that UNx leads to a positive feedback loop and hence to autocrine stimulation of VEGFR-2.39

Mesangial proliferation induced by Fk-sel therapy was associated with alterations in expression of PDGF/PDGFR, which are potent mitotic factors for mesangial cells.40 This finding is consistent with previous report.41 Notably, VEGF-A is capable of signaling through PDGFRs,42 and so our VEGF-A mutant might directly stimulate PDGF pathways in our animals, leading to alterations in mesangial cell biology. Future studies are required to address this issue.
The role of VEGFR2 in podocytes is controversial. Veron et al. documented that overexpression of VEGF-A in podocytes stimulates VEGFR2 expression in autocrine fashion, whereas our experiments showed that VEGFR2 stimulation did not affect podocyte structure. The most likely explanation for this discrepancy is that VEGF-A was locally overexpressed in podocytes themselves in the Veron et al. study, but Flk-sel levels were increased in the circulation in the present study. The concentration of Flk-sel could presumably be reduced when it reaches podocyte cells. Alternatively, our findings are supported also by a recent study showing that VEGFR2-knockout podocytes do not lead to any glomerular abnormalities in mice up to 6 months of age, indicating that podocyte VEGFR2 is biologically inactive.

A striking finding in the present study was that Flk-sel overexpression led to expression of α-SMA in some epithelial cells in damaged tubules. This was confirmed by our in vitro study, which showed that VEGF-A caused a reduction of the E-cadherin expression (a marker of epithelial cells) and an induction of α-SMA expression (a marker of mesenchymal cells), suggesting that VEGF might cause a phenotypic change in tubular cells. Caution is advised in interpreting these data, however, because recent studies have challenged the existence of this epithelial-mesenchymal transition in kidney disease.

In opposition to our predictions, and despite the fact that inflammatory reactions after VEGF-stimulation are thought to be attributed to VEGFR1, not VEGFR2, selective VEGFR2 stimulation elevated macrophage infiltration as assessed by F4/80 and CD68 staining, regardless of eNOS expression. The renal macrophage infiltration observed in the present study was unlikely to be due to direct VEGFR2 stimulation, but probably was due to an indirect effect from other cytokines that may be expressed in injured kidneys.

We found that stimulation of VEGFR2 caused renal injury, but without aggravation of albuminuria. This discordance between urine albumin excretion and renal function has been found also in several clinical studies. The ONTARGET study showed that stronger inhibition of the renin-angiotensin system accelerated renal disease progression, whereas urinary protein excretion was reduced in injured kidneys.

In conclusion, the present study documented that VEGFR2 stimulation before renal insult can predispose to an acceleration of renal disease, especially in the presence of endothelial NO deficiency.

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