Soluble Flt-1 gene therapy ameliorates albuminuria but accelerates tubulointerstitial injury in diabetic mice

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Am J Physiol Renal Physiol 298: F609–F616, 2010. First published December 16, 2009; doi:10.1152/ajprenal.00377.2009.—VEGF is recognized as a major mediator in the development of diabetic nephropathy. Soluble Flt-1 (sFlt-1) is the endogenous inhibitor of VEGF, and recently genetic overexpression of sFlt-1 in the podocyte was shown to be protective in murine diabetic nephropathy. In this study, we performed a translational study to determine whether an intramuscular gene transfer of sFlt-1 can prevent the progression of renal disease in diabetic db/db mice. Adeno-associated virus-1 (AAV1) encoding human sFlt-1 in two different doses was intramuscularly administrated in db/db and wild-type mice. The sFlt-1-AAV1 treatment significantly increased serum sFlt-1 level at 4 and 8 wk. A dose that was developed in this study caused minimal abnormalities in normal mice but reduced albuminuria in diabetic db/db mice. In renal histology, sFlt-1 treatment at this dose had minimal effects on mesangial expansion in diabetic mice, whereas podocyte injury was significantly improved, at 8 wk. Unfortunately, tubulointerstitial injury was markedly exacerbated by sFlt-1 treatment in association with a reduction in endogenous VEGF expression and peritubular capillary loss. In conclusion, gene therapy with sFlt-1-AAV1 protects podocytes but accelerates tubulointerstitial injury in diabetic db/db mice. These data suggest systemic overexpression of sFlt-1 will not likely be useful for treating diabetic nephropathy.

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nonameric polyglycine (34). A total of 6 groups consisting of 10 mice/group were studied: 1) control C57BLKS/J mice with empty vector-AAV1, 2) control mice with low-dose sFlt-1-AAV1 [3.3×10⁹ viral particles (VP)], 3) control mice with high-dose sFlt-1-AAV1 (1.0×10¹⁰ VP), 4) db/db mice with empty vector-AAV, 5) db/db mice with low-dose sFlt-1-AAV1, and 6) db/db mice with high-dose sFlt-1-AAV1. AAV1 was intramuscularly injected into calf muscle once at the beginning of study. Systolic blood pressure (BP) was assessed using a tail-cuff sphygmomanometer (Visitec BP2000; Visitech Systems, Apex, NC). Blood and urine samples were collected at 1 and 2 mo after injection. Serum creatinine, creatinine clearance, and urinary albumin-to-creatinine ratio were measured with Albwell M (Exocell, Philadelphia, PA) and/or liquid creatinine assay (Bioquant, San Diego, CA) as described previously (32).

sFlt-1 and VEGF in serum and urine. Serum sFlt-1, urinary sFlt-1, and urinary VEGF levels were measured at 8 wk using a human quantikine ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions.

Western blot analysis. Mouse kidney tissues were snap-frozen in liquid nitrogen for protein isolation. Western blot analysis was performed as described previously (22). The blots were subsequently incubated with a monoclonal rabbit anti-VEGFR1 antibody (Epitomics, Burlingame, CA), followed by incubation with peroxidase-conjugated anti-rabbit IgG (DakoCytomation, Carpinteria, CA). Receptor phosphorylation was detected using a polyclonal rabbit anti-phospho-Flt-1 (pY1213) antibody (Millipore, Temecula, CA). Proteins were assessed using an enhanced chemiluminescence detection system (Amersham Pharmacia, Piscataway, NJ). The density of each band was measured using the public domain NIH Image program.

Renal histology. Kidneys were fixed in neutral buffered formalin (10%) and embedded in paraffin, and 2-μm sections were stained with periodic acid-Schiff (PAS) for immunohistochemistry. A polyclonal rabbit anti-human fibronectin antibody (1:200; Sigma-Aldrich, St. Louis, MO), a polyclonal rabbit anti-mouse collagen IV antibody (Chemicon International, Temecula, CA), a polyclonal goat anti-human collagen III antibody (1:100; Southern Biotechnology Associates, Birmingham, AL), a polyclonal rabbit anti-mouse osteopontin antibody (1:400; Cosmo Bio, Tokyo, Japan), a polyclonal goat anti-rat collagen IV antibody (Chemicon International, Temecula, CA), a polyclonal goat anti-mouse collagen III antibody (1:100; Southern Biotechnology Associates, Birmingham, AL), a polyclonal rabbit anti-mouse fibronectin antibody (1:200; Sigma-Aldrich, St. Louis, MO), a polyclonal rabbit anti-human fibronectin antibody (1:200; Sigma-Aldrich, St. Louis, MO), and a polyclonal goat anti-rat CD31 antibody (BD Pharmingen, San Jose, CA) were used for immunohistochemistry. Color was developed using 3,3′-diaminobenzidine (Dako, Carpinteria, CA). Negative controls were performed by replacing primary antibodies with species-matched antibodies. Quantification of staining was performed in glomerular and renal cortical fields with the AxioVision image analysis computer program (Carl Zeiss, Thornwood, NY) in a blinded manner by two independent observers.

To detect endothelial cell proliferation, we performed double immunostaining with antibodies for the cell proliferation marker Ki-67 (Abcam, Cambridge, MA) and the endothelial cell marker CD34. Fast red (Biocare Medical, Concord, CA) was used for double staining. Double-positive cells for CD34 and Ki-67 were counted by two independent investigators in a blinded manner.

Table 1. General characteristics at 8 wk after AAV shot

<table>
<thead>
<tr>
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<th>Control Mice</th>
<th>db/db Mice</th>
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<tbody>
<tr>
<td></td>
<td>No treatment</td>
<td>Low sFlt-1</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>26.4±1.1</td>
<td>25.8±0.7</td>
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<tr>
<td>Blood glucose, mg/dl</td>
<td>99±9.1</td>
<td>105±15</td>
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<tr>
<td>Blood pressure, mmHg</td>
<td>113±5.9</td>
<td>119±4.5</td>
</tr>
<tr>
<td>BUN, mg/dl</td>
<td>214±5.5</td>
<td>22.2±3.9</td>
</tr>
<tr>
<td>Serum creatinine, mg/dl</td>
<td>0.10±0.03</td>
<td>0.12±0.05</td>
</tr>
<tr>
<td>Creatinine clearance, 10⁻²</td>
<td>1.4±0.49</td>
<td>1.2±0.70</td>
</tr>
<tr>
<td>Urine albumin, μg/16 h</td>
<td>6.1±2.8</td>
<td>8.3±3.5</td>
</tr>
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</table>

Data are means ± SD (n = 10). aP < 0.001; bP < 0.05; cP < 0.005 vs. control mice with no treatment. dP < 0.01; eP < 0.05 vs. db/db mice with no treatment.
Morphological assessment. For each biopsy, 50 glomeruli were examined. The mesangial area was determined by assessing the PAS-positive and nuclei-free area in the mesangium, as previously described (22). The glomerular area was also measured by outlining the glomerular tuft. Their areas were measured with the AxioVision image analysis computer program (Carl Zeiss). The degree of mesangiolysis was assessed as the number of glomeruli with dissolution of the mesangial matrix, as previously described (32). For analysis of the tubulointerstitium, the percentage of atrophic tubules (i.e., tubular dilation, detachment of tubular epithelial cells, and condensation of tubular nuclei) was assessed by scoring 800 renal cortical tubules in randomly selected fields for each subject (23). All quantifications were performed in a blinded manner by two independent investigators.

Statistical analysis. All values are means ± SD. Statistical analysis was performed with unpaired, two-tailed Student’s t-tests for single comparisons or with ANOVA for multiple comparisons. Post hoc least significant difference (LSD) tests were carried out if the initial ANOVA was significant. A P value < 0.05 was taken to indicate a significant difference.

RESULTS

General characteristics. Studies were performed with both wild-type and diabetic db/db mice. In wild-type mice, the administration of sFlt-1 was associated with the development of mild albuminuria and elevated blood pressure at the high but not the low dose (Table 1). These data are consistent with previous studies showing that the administration or overexpression of sFlt-1 to rodents can induce renal injury (7, 38).
Both low and high doses of AAV1-sFlt-1 were also administered to db/db mice. The treatment was well tolerated and did not alter body weight or blood sugar compared with control db/db mice. Importantly, neither dose altered blood pressure. Both treatments resulted in significant reductions in albuminuria, consistent with a beneficial effect. However, renal function (as evidenced by serum creatinine and creatinine clearance) tended to be worse, although it did not reach significance. An unexpected finding was that both doses were associated with an increase in blood urea nitrogen levels (Table 1).

**VEGF signaling blockade by s-Flt-1-AAV1 treatment.** We next sought to determine whether the expression of sFlt-1 could functionally block VEGF signaling. Serum sFlt-1 level was undetectable in mice with empty vector-AAV1. However, intramuscular injection of sFlt-1-AAV1 resulted in a significant elevation in serum sFlt-1 level at 4 wk, which was sustained by 8 wk in both control and db/db mice (Fig. 1A). Serum sFlt-1 level was dose-dependently increased by AAV1 treatment, with high-dose sFlt-1-AAV1 causing a fourfold higher level of sFlt-1 compared with low-dose sFlt-1-AAV1. Urinary sFlt-1 was not detected in both types of mice. To confirm whether the elevated serum levels of sFlt-1 could block VEGF function in the kidney, we examined the phosphorylation of Flt-1 (which is a marker of Flt-1 signaling) in the renal cortex of sFlt-1-overexpressing mice. As shown in Fig. 1, B and C, Flt-1 phosphorylation was significantly sup-

![Fig. 3. Tubulointerstitial changes. A: PAS staining in tubulointerstitium from control mice with empty vector-AAV1 (a), low-dose sFlt-1-AAV1 (b), and high-dose sFlt-1-AAV1 (c) and from db/db mice with empty vector-AAV1 (d), low-dose sFlt-1-AAV1 (e), and high-dose sFlt-1-AAV1 (f). db/db mice with empty vector show subtle tubulointerstitial injury. sFlt-1 treatments further deteriorated tubulointerstitial injury. Bar, 50 μm. B: quantitative analysis for tubular injury with PAS staining. C: immunohistochemistry for collagen III (a–d) and osteopontin (arrows; e–h). Compared with control mice with empty vector-AAV1 (a and e), db/db mice with empty vector show an increase in interstitial collagen III deposition (b) and tubular osteopontin expression (f). Both low-dose (c and g) and high-dose sFlt-1-AAV1 treatments (d and h) significantly accelerated interstitial collagen III deposition (c and d) and tubular osteopontin expression (g and h). Bar, 50 μm. D and E: quantitative analysis for collagen III (D) and osteopontin (E). Open bars, empty vector-AAV1; filled bars, low-dose sFlt-1-AAV1; shaded bars, high-dose sFlt-1-AAV1. Data are means ± SD (n = 10). aP < 0.001; bP < 0.05; cP < 0.005.](image-url)
pressed by both low and high doses of sFlt-1-AAV1 treatment in these animals.

Glomerular injury. Compatible with previous reports (37), db/db mice developed mesangial expansion. Treatment with sFlt-1-AAV1, however, did not block this mesangial injury in db/db mice (Fig. 2A). Similarly, immunohistochemistry showed that mesangial matrix deposition (fibronectin and collagen IV) was also not reduced by sFlt-1 treatment in db/db mice (Fig. 2, B–D).

We next examined podocyte injury. Podocyte density, elucidated by staining for WT-1, was significantly reduced in db/db mice compared with control mice (Fig. 2E). The reduction of WT-1-positive cells was prevented by sFlt-1 treatment (Fig. 2E). Expression of desmin (a sign of podocyte stress) was also increased in db/db mice, and the expression was prevented by sFlt-1 treatment (Fig. 2F). In contrast, sFlt-1 treatment did not change desmin expression in wild-type mice.

Tubulointerstitial injury. PAS staining documented that diabetes caused a subtle but significant tubular injury in db/db mice, as predominantly evidenced by ballooning of tubules (Fig. 3A). Interestingly, both low and high doses of sFlt-1-AAV1 caused greater injury in db/db mice, as evidenced by more severe tubular dilation, atrophy, and epithelial cell detachment. In contrast, sFlt-1 treatment had no effect on tubules in control mice (Fig. 3, A and B). Consistent with these findings, interstitial collagen III deposition was also increased in db/db mice and was exacerbated by sFlt-1 treatments (Fig. 3, C and D). Similarly, tubular damage, as evaluated by osteopontin expression (36), was also increased in db/db mice compared with control mice and was further increased by sFlt-1 treatment in db/db mice (Fig. 3, C and D). In contrast, control mice overexpressing sFlt-1 did not show any increase in collagen III deposition or tubular osteopontin expression with sFlt-1 treatment.

Fig. 4. CD34 expression and endothelial proliferation in glomeruli and peritubules. A: immunohistochemistry for CD34 expression in tubulointerstitium of db/db mice with empty vector-AAV1 at low (a) and high magnification (b) and with high-dose sFlt-1-AAV1 treatment at low (c) and high magnification (d). B: percent positive signal for CD34 in tubulointerstitium per mm². C: percent positive signal for CD34-positive endothelial cells in glomerulus. D: representative positive cells for both CD34 (red) and Ki-67 (brown) in proliferating endothelial cells in glomerular capillary (a) and peritubular capillary (b). E: number of double-positive cells in glomerulus. F: number of double-positive cells in peritubular capillary. Open bars, empty vector-AAV1; filled bars, low-dose sFlt-1-AAV1; shaded bars, high-dose sFlt-1-AAV1. Data are means ± SD (n = 10). *P < 0.001; †P < 0.01; ‡P < 0.05.
**Glomerular and peritubular capillaries.** Since VEGF is a key angiogenic and trophic factor for endothelial cells, we next examined the effects of the sFlt-1 treatments on the glomerular and peritubular capillaries. Consistent with previous reports (17), immunohistochemistry for CD34 demonstrated that endothelial cells were increased in the glomerular and peritubular capillaries of db/db mice. Interestingly, peritubular capillary endothelial cells were significantly reduced by these treatments (Fig. 4, A and B). In contrast, glomerular endothelial cell density was not altered by sFlt-1 treatment in either control or db/db mice (Fig. 4C). Next, the number of proliferating endothelial cells was examined by double staining of CD34 with Ki-67, a marker of cell proliferation. Glomerular endothelial cell proliferation was significantly increased in glomeruli of db/db mice but was not prevented by sFlt-1 treatment (Fig. 4D). Endothelial cell proliferation was also increased in the peritubular capillaries of db/db mice and, in contrast to the glomeruli, was significantly blocked by sFlt-1-AAV1 treatment (Fig. 4E).

**VEGF expression in the kidney.** It is known that VEGF is constitutively expressed in both podocytes and tubular epithelial cells, where they have important trophic roles on the adjacent endothelium. Glomerular VEGF expression was elevated in db/db mice compared with control mice (Fig. 5A), consistent with previous reports (17, 39). However, sFlt-1 treatment did not change the VEGF level in glomeruli (Fig. 5A). Tubular VEGF expression was also increased in db/db mice. However, the expression was significantly suppressed by sFlt-1 treatment (Fig. 5B). Urinary VEGF level was also higher in db/db mice, but it was not altered by sFlt-1 treatment in this study (Fig. 5C).

**DISCUSSION**

Previous studies have documented that blocking VEGF with sFlt-1 overexpression can cause renal disease, including glomerular thrombotic microangiopathy (25, 28, 38). This evidence indicates that inhibiting physiological VEGF is deleterious, and therefore VEGF inhibition as a therapeutic approach should target only pathological VEGF. We have demonstrated that two different doses of AAV1 tested in this study significantly raised sFlt-1 levels in the systemic circulation and inhibited VEGF signaling in the kidney, as noted by a reduction in Flt-1 phosphorylation in these mice. Importantly, neither dose of sFlt-1 chosen was found to cause significant glomerular or tubulointerstitial injury in wild-type mice, suggesting that we succeeded in identifying a dose of sFlt-1 that appeared to be safe for wild-type mice and that could be tested in our diabetic model.

The fact that sFlt-1 treatment reduced tubular VEGF expression along with blocking Flt-1 phosphorylation suggests that tubular VEGF expression could be regulated in an autocrine loop of VEGF and VEGFRs (11, 12, 40). Similarly, several in vitro studies have also indicated that a VEGF and VEGFR autocrine loop may exist in the podocyte (2, 3, 11, 12). However, controversy still remains as to whether podocytes truly express VEGFR1 in vivo. Although we found that sFlt-1 treatment had protective effects on the podocytes, as noted by preserved podocyte (WT-1) number, it is still unknown whether this beneficial effect was due to a direct inhibition of VEGFR1 phosphorylation on the podocyte. Alternatively, several studies have suggested that the podocyte loss in diabetic nephropathy may be due to increased angiotensin II levels (15), and VEGF is known to be a potent stimulus for angiotensin II production in diabetic glomeruli (18, 29). Hence, it remains possible that the effect of VEGF inhibition to prevent podocyte loss is an indirect effect mediated by a reduction in glomerular angiotensin II levels.

Interestingly, overexpression of systemic sFlt-1 failed to prevent the mesangial expansion in this study. Previous studies have demonstrated that VEGF derived from podocytes contributes to the formation of the mesangium in the developing kidney (6, 8). In diabetic animals, VEGF inhibition can reduce glomerular volume in diabetic db/db mice (5, 9, 10). These studies suggest that VEGF mediates mesangial expansion in the diabetic condition. However, the latter study did not examine mesangial area, and therefore the role of VEGF on mesangial expansion is still unclear. Given the fact that abnormal angiogenesis is also known to be a component of diabetic mesangial expansion (16, 33), it is possible that blocking VEGF may reduce glomerular capillary abnormalities but may not block mesangial expansion. Likewise, our study demonstrated that mesangial expansion was not improved
despite Flt-1 phosphorylation being significantly blocked by sFlt-1-AAV treatment. Other factors such as high glucose or transforming growth factor-β1 also could be responsible for mesangial expansion in this model (32, 41).

Another finding in this study was that sFlt-1 treatment caused endothelial cell loss in peritubular capillaries but not in glomerular capillaries. The differential effect of sFlt-1 therapy on glomerular vs. peritubular capillaries might be explained by differences in VEGF levels in the glomeruli and tubules. Despite sFlt-1 treatment, glomerular VEGF expression was preserved, whereas tubular VEGF was significantly reduced. Since VEGF is essential for maintaining glomerular and peritubular capillary density (19), a high level of VEGF might be able to maintain an increased glomerular capillary number, whereas a reduction in VEGF in the tubules might result in a decrease of peritubular capillary density.

If this is the case, one might question why glomerular VEGF was preserved despite sFlt-1 treatment, as opposed to tubular VEGF. One possibility is that it might reflect differences in access, but we believe that an inhibition of VEGF by a circulating inhibitor should be uniform throughout the circulation; however, the local effects could relate to the concentrations of VEGF in adjacent cells. In the tubulointerstitium, the tubules are expressing most of the VEGF, but it is known that they are exquisitely dependent on peritubular capillaries for viability. If the peritubular capillary is injured, the tubules undergo ischemic damage and lose their VEGF. In contrast, the podocyte may be able to maintain its VEGF and viability even when the glomerular endothelium is damaged. In a study by Kim et al. (21), it was shown that direct injury to the glomerular and peritubular capillary endothelium with an anti-endothelial cell antibody led to a transient injury in the glomerulus but to persistent injury in the tubules, and this was associated with a loss of VEGF in the injured tubules, whereas glomerular VEGF levels were maintained.

In our model, we have found that diabetes can be associated with excessive angiogenesis and inflammation, consistent with the known effects of an elevated VEGF, especially under conditions of endothelial dysfunction (31, 32). However, a loss of VEGF is also known to cause peritubular capillary loss and increase the risk for fibrosis in both nondiabetic and diabetic renal injury (20, 30). In our study utilizing sFlt-1 therapy, we have observed evidence for both mechanisms. Whereas tubulointerstitial injury was associated with increased endothelial cells and inflammation, a reduction of capillary by sFlt-1 was associated with fibrosis. Thus these studies emphasize how both a lack and an excess of VEGF may contribute to renal disease.

The sFlt-1-AAV used in this study was composed of extracellular domain of Flt-1 and human IgG1-Fc region. Although we could not exclude the possibility that some of the observed effects may be due to the IgG1-Fc region, it is more likely that the antiangiogenic effects were mediated by the sFlt-1 protein component.

In conclusion, we have confirmed a beneficial effect of blocking VEGF in podocyte injury in experimental diabetic nephropathy. Unfortunately, we have demonstrated that such treatment has significant negative effects on the tubulointerstitium, resulting in peritubular capillary loss, tubular injury, and interstitial collagen deposition, and worsens renal function. These studies suggest that sFlt-1 treatment is unlikely to be beneficial in diabetic nephropathy and that alternative approaches to treating diabetic renal disease are needed.

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DISCLOSURES

No conflicts of interest are declared by the authors.

REFERENCES


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