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

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Vascular endothelial growth factor; adeno-associated virus serotype 1

VEGF is recognized as a major mediator in the development of diabetic nephropathy (4). VEGF-A is considered a mediator of diabetic nephropathy (4). VEGF-A is increased in experimental diabetic nephropathy (7), hypertension (1), and antibody-mediated renal injury (13). Soluble Flt-1 (sFlt-1) is an endogenous inhibitor of VEGF (14). VEGF-A is increased in experimental diabetic nephropathy (7), hypertension (1), and antibody-mediated renal injury (13). Soluble Flt-1 (sFlt-1) is an endogenous inhibitor of VEGF given that it is a variant form of VEGF receptor 1 (VEGFR1) that lacks the transmembrane and intracellular domains of VEGFR1 yet binds to VEGF with the same affinity as VEGFR1 and therefore blocks VEGF function. Ku et al. (24) recently generated a mouse model in which sFlt-1 is specifically overexpressed in podocytes to demonstrate that site-specific overexpression of sFlt-1 in the podocyte significantly blocked the development of diabetic nephropathy in streptozotocin-induced diabetic mice without any adverse effects. This suggests that sFlt-1 could be an ideal means to block VEGF-A in diabetic nephropathy. Although these studies elegantly demonstrate that VEGF is a mediator of diabetic nephropathy, it is unlikely that overexpression of sFlt-1 in podocyte will be an available therapeutic option in the near future. Hence, the next step toward taking this treatment to clinic would be to test whether systemic treatment with sFlt-1 could prevent the development of diabetic nephropathy in experimental diabetic nephropathy.

Adeno-associated virus (AAV) is a small (20 nm) virus and belongs to the genus Dependovirus, which infects humans and some other primate species (14). AAV is known to have very low immunogenicity and a lack of pathogenicity. AAV vectors have been found to promote sustained gene expression in a variety of tissues such as muscle, eye, brain, liver, and lung in humans and animal models (26, 27, 35). Because of these advantages, AAV has emerged as an attractive vector for gene therapy (14).

We performed a translational study in which we used AAV1 to increase the systemic level of sFlt-1 in db/db mice. Because excessive inhibition of VEGF could result in negative renal effects, we gave two different doses (both a low and a high dose) of AAV1 to determine whether there is a therapeutic window for treatment.

METHODS

Animals and experimental design. Experiments were performed following protocol approval by the Animal Care and Use Committee of the University of Florida. Diabetic db/db (BKS.Cg-m¹⁺/+Lepdb/J) mice and age-matched background strain C57BLKS/J mice (8 wk old, male) were purchased from the Jackson Laboratory (Bar Harbor, ME). All mice were housed in a specific pathogen-free animal facility at the University of Florida. AAV1 encoding human sFlt-1 was kindly provided by Dr. William W. Hausswirth (34). The sFlt-1 used in this study contains a Flt-1 domain 2 linked to a human IgG1-Fc region by
Viral particles (VP), 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 × 10^9 viral particles (VP)], 3) control mice with high-dose sFlt-1-AAV1 (1.0 × 10^10 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 (Visitech 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 auto-phosphorylation was detected using a polyclonal rabbit anti-phospho-Flt-1 (pY1213) antibody (Millipore, Temecula, CA). Proteins were visualized with 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 VEGF antibody (1:100; R&D Systems), and a polyclonal rat anti-mouse collagen III 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.

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>
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<tr>
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<th>Control Mice</th>
<th>db/db Mice</th>
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<tr>
<td></td>
<td>No treatment</td>
<td>Low sFlt-1</td>
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<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>
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<tr>
<td>BUN, mg/dl</td>
<td>21.4 ± 5.5</td>
<td>22.2 ± 3.9</td>
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<tr>
<td>Serum creatinine, mg/dl</td>
<td>0.10 ± 0.03</td>
<td>0.12 ± 0.05</td>
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<tr>
<td>Creatinine clearance, 10^-2</td>
<td>1.4 ± 0.49</td>
<td>1.2 ± 0.70</td>
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<tr>
<td>Urine albumin, µg/16 h</td>
<td>6.1 ± 2.8</td>
<td>8.3 ± 3.5</td>
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Data are means ± SD (n = 10). *P < 0.001; #P < 0.05; †P < 0.005 vs. control mice with no treatment. ‡P < 0.01; §P < 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). *P < 0.001; **P < 0.05; ***P < 0.005.
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: representat-
tive positive cells for both CD34 (red) and Ki-67
(brown) in proliferating endothelial cells in glomeru-
lar capillary (a) and peritubular capillary (b). E: num-
ber 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 also have 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 diabetic 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.

![Fig. 5. Effect of sFlt-1 treatment on VEGF expression in the kidney. A: percent positive signal for glomerular VEGF expression. B: percent positive for tubular VEGF expression per mm² in cortex. C: amount of VEGF excretion for 16 h in urine. 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 < 0.05.](http://ajprenal.physiology.org/10.220.32.247)
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


