Amelioration of renal alterations in obese type 2 diabetic mice by vasohibin-1, a negative feedback regulator of angiogenesis

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Am J Physiol Renal Physiol 300: F873–F886, 2011. First published January 12, 2011; doi:10.1152/ajprenal.00503.2010.—The involvement of VEGF-A as well as the therapeutic efficacy of angiogenesis inhibitors in diabetic nephropathy have been reported. We recently reported the therapeutic effects of vasohibin-1 (VASH-1), an endogenous angiogenesis inhibitor, in a type 1 diabetic nephropathy model (Nasu T, Maeshima Y, Kinomura M, Hirokoshi-Kawahara K, Tanabe K, Sugiyama H, Sonoda H, Sato Y, Makino H. Diabetes 58: 2365–2375, 2009). In this study, we investigated the therapeutic efficacy of VASH-1 on renal alterations in obese mice with type 2 diabetes. Diabetic db/db mice received intravenous injections of adenoviral vectors encoding human VASH-1 (AdhVASH-1) and were euthanized 8 wk later. AdhVASH-1 treatment resulted in significant suppression of glomerular hypertrophy, glomerular hyperfiltration, albuminuria, increase in the CD31+ glomerular endothelial area, F4/80+ monocyte/macrophage infiltration, the accumulation of type IV collagen, and mesangial matrix. An increase in the renal levels of VEGF-A, VEGFR-2, transforming growth factor (TGF)-β1, and monocyte chemotactrant protein-1 in diabetic animals was significantly suppressed by AdhVASH-1 (immunoblotting). AdhVASH-1 treatment significantly recovered the loss and altered the distribution patterns of nephrin and zonula occludens (ZO-1) and suppressed the increase in the number of fibroblast-specific protein-1 (FSP-1) and desmin+ podocytes in diabetic mice. In vitro, recombinant human VASH-1 (rhVASH-1) dose dependently suppressed the upregulation of VEGF induced by high ambient glucose (25 mM) in cultured mouse podocytes. In addition, rhVASH-1 significantly recovered the mRNA levels of nephrin and the protein levels of ZO-1 and podocin and suppressed the increase in protein levels of desmin, FSP-1, Snail, and Slug in podocytes under high-glucose condition. Taken together, these results suggest the potential use of VASH-1 as a novel therapeutic agent in type 2 diabetic nephropathy mediated via antiangiogenic effects and maintenance of podocyte phenotype in association with antiproteinuric effects.

podocyte; proteinuria

diabetic nephropathy is complicated in 30–40% of patients with type 2 diabetes and is the most common pathological disorder predisposing end-stage renal disease in Japan and the Western world. Glomerular hyperfiltration, glomerular and tubular hypertrophy, microalbuminuria, and thickening of the glomerular basement membrane (GBM) are observed in the early stage of diabetic nephropathy. Then, expansion of the mesangial extracellular matrix (ECM) and overt proteinuria are observed, eventually leading to glomerulosclerosis and tubulointerstitial fibrosis (27). The involvement of various factors and cytokines including the renin-angiotensin-aldosterone system, oxidative stress, insulin-like growth factor-I, monocyte chemotactrant protein-1 (MCP-1), transforming growth factor-β1 (TGF-β1), protein kinase C and advanced glycation end products (AGE) in diabetic nephropathy has been reported (4, 36).

Angiogenesis is associated with a number of pathological conditions including tumor growth and diabetic retinopathy (13). Vascular endothelial growth factor-A (VEGF-A), a potent stimulator of angiogenesis, promotes endothelial cell proliferation, migration, and endothelial cell tube formation (11) and also induces vascular permeability (9).

Previous studies have demonstrated an increased glomerular filtration surface area in diabetic nephropathy in association with the formation of new glomerular capillaries (14, 30) and a slight elongation of the preexisting capillaries (14, 30), analogous to the changes in pathological diabetic retinopathy (25). In addition, the increase in the levels of VEGF-A and the receptor of VEGF-A, VEGFR-2, has been reported in diabetic nephropathy models (7, 41). The therapeutic efficacies of anti-VEGF-A strategies (i.e., neutralizing antibodies and a receptor tyrosine kinase inhibitor) (8, 12, 38) as well as amelioration of diabetic glomerular alterations in mice with inducible podocyte-specific overexpression of soluble flt-1, an antagonist of VEGF-A (21), have further demonstrated the potential involvement of VEGF-A in the progression of diabetic nephropathy.

Recently, the therapeutic effects of angiogenesis inhibitors such as tumstatin peptide, endostatin peptide, angiotatin, pigment epithelium derived factor (PEDF), and NM-3 (17, 18, 25, 43, 48, 50) in diabetic nephropathy models have been reported by others and us.

Vasohibin-1 (VASH-1), an endogenous angiogenesis inhibitor, was identified from a microarray analysis assessing genes upregulated by VEGF-A in endothelial cells (45). Human VASH-1 protein is composed of 365 amino acid residues (45). VASH-1 is induced by representative angiogenic factors such as VEGF-A and fibroblast growth factor 2 (FGF-2) (45). VASH-1 regulates proliferation and migration of endothelial cells in an autocrine manner and thus is considered to serve as a negative feedback regulator of angiogenesis. In addition, VASH-1 inhibits lymphangiogenesis and lymph node metastasis of tumors (16). VASH-1 does not contain a classic signal

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sequence, and a small vasohibin-binding protein (SVBP) serves as a secretory chaperon for VASH-1 and contributes to the antiangiogenic effects of VASH-1 (39). To date, cell surface receptors for VASH-1 have not been reported. The therapeutic efficacies of VASH-1 on tumor growth, atherosclerosis, and proliferative retinopathy models have been reported (37, 45, 49). We recently reported the therapeutic effects of adenoviral transfer of VASH-1 (AdhVASH-1) in a mouse type 1 diabetic nephropathy model (29). Renoprotective effects of VASH-1 were mediated via its direct effects on mesangial cells as well as glomerular endothelial cells, suggesting the ability of VASH-1 beyond the “antiangiogenic factor.”

Nephrin, a glomerular podocyte protein, is crucial for maintaining the integrity of the interpodocyte slit membrane structure and an intact filtration barrier. In diabetic nephropathy, loss of intact podocyte-specific expression patterns of slit membrane-associated proteins such as nephrin (2), zonula occludin (ZO)-1, P-cadherin, and the expression of mesenchymal markers such as desmin, fibroblast-specific protein-1 (FSP-1) as well as Snail, a transcription factor involved in epithelial-to-mesenchymal transition (EMT), in association with the loss of glomerular filtration barrier function, has been reported (22, 47).

In the present study, we demonstrate the therapeutic efficacy of VASH-1 in ameliorating renal alterations in type 2 diabetic db/db mice. Treatment with AdhVASH-1 markedly suppressed characteristic alterations of diabetic nephropathy without affecting metabolic parameters. These effects were associated with the regulation of VEGF-A signals, inhibitory effects on chemokine and the direct protective effects of VASH-1 on podocytes, potentially leading to the amelioration of albuminuria.

MATERIALS AND METHODS

Adenoviral vectors. A replication-defective adenoviral vector encoding human VASH-1 was prepared as previously described (45). A replication-defective adenovirus vector encoding the Escherichia coli β-galactosidase (AdLacZ), which is identical to AdhVasohibin-1 (AdhVASH-1), except for the inserted cDNA, was used as the control (45). Adenoviral vectors were expanded in human embryonic kidney cell line 293 and purified by cesium chloride ultracentrifugation as described previously (19). The purified viruses were dialyzed against PBS with 10% glycerol and stored at −80°C until use. The viral concentration and the viral titer were determined as previously described (45).

Experimental protocols. The experimental protocol was approved by the Animal Ethics Review Committee of Okayama University. Adult male db/db mice (BKS.Cg-db/db Jcl; Clea Japan, Osaka, Japan) and their age-matched nondiabetic db/m littermates (BKS.Cg-db/+ m Jcl; Clea Japan) were used. Mice were fed a standard pellet laboratory diet and were provided with water ad libitum. The body weight and the individual 24-h food consumption were measured. At 8 wk after initiation of treatment, individual 24-h urine sample collection was performed using metabolic cages. Nonfasting blood samples were drawn from the retroorbital venous plexus using heparinized capillary tubes under anesthesia at the time of euthanasia. Kidney, liver, and heart weight was measured just after euthanasia.

Blood and urine examination. Blood glucose was measured in tail-vein blood, and urine was tested for ketone bodies and glucose by Okayama Medical Laboratories (Okayama, Japan). Serum and urinary creatinine levels were measured by HPLC. Urinary albumin concentration was measured by ELISA (Bethyl Laboratories, Montgomery, TX) following the manufacturer’s instructions. Results were normalized to the urinary creatinine levels and expressed as the urinary albumin/creatinine ratio (UACR). The creatinine clearance (CrCr) was calculated and expressed as milliliters per minute per 100 grams of body weight. Serum levels of mouse insulin were determined by ELISA using an ultrasensitive rat insulin ELISA kit and mouse insulin standard (Morinaga, Yokohama, Japan) following the manufacturer’s instructions. According to the manufacturer’s technical information, mouse insulin can be measured in combination with a mouse insulin standard due to a high homology among mammalian animals. All samples were examined in duplicate, and mean values of individual sera were utilized for statistical analysis. The intra- and interassay coefficients of variation for the insulin assays were <5% and <10%, respectively.

Measurement of blood pressure. Arterial blood pressure was measured before euthanasia using a programmable sphygmomanometer (BP-98A; Softron, Tokyo, Japan) by the tail-cuff method as described previously (15).

Histological analysis. At 8 wk after the start of treatment, the kidneys were removed, fixed in 10% buffered formalin, and embedded in paraffin. Sections (4-μm) were stained with periodic acid-Schiff to evaluate microscopic observation. The mean glomerular tuft volume (GTV) was determined from the mean glomerular cross-sectional tuft area (GA) as described previously (17, 46, 48). Twenty glomeruli from each cortical area were observed, images were taken and analyzed by using Lumina Vision software (Mitani, Fukui, Japan) to determine the mean GA, GTV was calculated as GTV = β/π × (GA)²/3, with β = 1.38, the shape coefficient for spheres, and k = 1.1, a size distribution coefficient (46).

The mesangial matrix index was defined as the proportion of the glomerular tuft occupied by the mesangial matrix, excluding nuclei. The mesangial matrix areas of 20 glomeruli in each kidney were analyzed and averaged. The mesangial areas were selected using Photoshop software (Adobe Systems, San Jose, CA), followed by analysis using Lumina Vision.

Immunohistochemistry. Immunofluorescence staining of CD31, type IV collagen, nephrin, and zonula occludins (ZO)-1 was performed using frozen sections as previously described (17, 29). The following antibodies were used as primary antibodies: a rat anti-mouse CD31 monoclonal antibody (Pharmingen, San Diego, CA); polyclonal rabbit anti-mouse type IV collagen antibody (Chemicon International, Temecula, CA); polyclonal guinea pig anti-nephin antibody (Fitzgerald, Concord, MA); and polyclonal rabbit anti-zonula occludens (ZO)-1 antibody (Zymed Laboratories, Carlsbad, CA). Briefly, frozen sections (4-μm) were fixed in cold (−10°C) acetone for 10 min and then air dried. The sections were blocked with 10% normal goat serum (Sigma, St. Louis, MO) for CD31, type IV collagen, and nephrin or Protein Block Serum-Free (Dako Cytomation, Carpinteria, CA) for ZO-1. The sections were incubated with...
primary antibodies as described above for 1 h. Then, the sections were washed, and incubated with secondary antibodies for 30 min at room temperature. After washing in PBS, sections were observed by a confocal laser fluorescence microscope (LSM-510; Carl Zeiss, Jena, Germany). The following FITC-conjugated antibodies were used as secondary antibodies: Alexa Fluor 546-labeled goat anti-rat IgG (CD31, Invitrogen, Carlsbad, CA); Alexa Fluor 488-labeled anti-rabbit IgG (type IV collagen and ZO-1, Invitrogen); and Alexa Fluor 488-labeled anti-guinea pig IgG (nephrin, Invitrogen).

The immunoreactivity of glomerular CD31 or type IV collagen was quantified as follows; color images were obtained as TIF files by LSM-510. The brightness of each image file was analyzed using

Fig. 1. A: immunoblot analysis. Immunoblots for human vasohibin-1 (VASH-1) and actin are shown. Each lane was loaded with 25 μg protein obtained from the serum samples or liver. Adenoviral vector (AdhVASH-1)-injected db/db mice exhibited significantly elevated serum VASH-1 (42 kDa) levels compared with the AdLacZ-injected db/db mice (16 wk of age). Similarly, enhanced protein levels of VASH-1 in the liver were observed in AdhVASH-1-treated mice compared with AdLacZ-treated db/db mice. Immunoblots for actin are shown to confirm equal loading. B: increase in urinary albumin/creatinine ratio (UACR) in db/db mice was significantly suppressed by treatment with AdhVASH-1 (Vas; 16 wk of age). *P < 0.05 vs. db/m. †P < 0.05 vs. db/db mice treated with vehicle buffer (Ve) or db/db mice treated with AdLacZ (LacZ). C: increase in creatinine clearance (Ccr) in db/db mice was partially suppressed by AdhVASH-1 (16 wk of age). *P < 0.05 vs. db/m. †P < 0.05 vs. Ve or LacZ. D–G: representative light microscopic appearance of glomeruli (periodic acid-Schiff staining; original magnification ×400) for db/m mice (D), db/db mice treated with either vehicle buffer (E), AdLacZ (F), or AdhVASH-1 (G) at 16 wk of age. H: increase in glomerular volume in db/db mice was diminished by treatment with AdhVASH-1. I: mesangial matrix index was defined as the proportion of the glomerular tuft occupied by the mesangial matrix area (excluding nuclei). Each column consists of means ± SE. *P < 0.01 vs. db/m. †P < 0.05 vs. Ve or LacZ; n = 6/group.
Lumina Vision. Image files (TIF) were inverted and opened in grayscale mode. Type IV collagen or CD31 indices were calculated using the following formula, [(X (density) × positive area (μm²))/glomerular total area (μm²)], where the staining density is indicated by a number from 0 to 256 in grayscale. In regard to peritubular capillary (PTC) density, the number of CD31+ peritubular capillaries in each high-power field was determined. The PTC density of 20 high-power fields in each kidney was analyzed and averaged.

To evaluate the staining pattern of nephrin and ZO-1, the following “re-distribution” score was used as described by Macconi et al. (24). A score was assigned to each individual glomerulus in the tissue section. The score 0, 0.5, and 1.0 were used, respectively, for continuous distribution and markedly discontinuous distribution. The final score per section was then calculated as the weighted mean: score = \[ \sum (N_i \times 0 + N_2 \times 0.5 + N_3 \times 1) \] (\(N_i + N_2 + N_3\)), where \(N_i\) (\(i = 1–3\)) is the number of glomeruli in each category.

Glomerular accumulation of monocytes/macrophages was determined by immunohistochemistry using rat anti-mouse F4/80 antibody (Serotec, Oxford, UK). Frozen sections were fixed in acetone for 10 min and exposed to H2O2 to eliminate endogenous peroxidase activity. Frozen sections were fixed in acetone for 10 min and exposed to H2O2 to eliminate endogenous peroxidase activity. Sections were then washed with PBS and exposed to a secondary antibody, horseradish peroxidase (HRP)-labeled goat anti-rat IgG (Chemicon) for 1 h. Diaminobenzidine was used as a chromogen. The kidney sections were then blocked with 10% goat serum for 30 min and incubated with biotinylated secondary antibody, and immunoperoxidase staining was carried out using a Vectastain ABC Elite reagent kit (Vector Labs, Burlingame, CA) as previously described (18, 48). Diaminobenzidine was used as a chromogen. The number of F4/80+ cells was determined by observing >20 glomeruli from each section. All slides were counterstained with hematoxylin. Normal goat IgG was used as a negative control.

For immunohistochemistry of desmin and FSP-1, formalin (10%)-fixed, paraffin-embedded sections (4-μm) were used. After deparaffinization, sections were incubated with goat polyclonal anti-desmin antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or rabbit polyclonal anti-S100A4 (FSP-1) antibody (Dako Cytomation) followed by incubation with biotinylated secondary antibody, and immunoperoxidase staining was carried out utilizing a Vectastain ABC Elite reagent kit (Vector Labs, Burlingame, CA) as previously described (18, 48). Diaminobenzidine was used as a chromogen. The number of desmin or FSP-1+ cells (podocytes) was determined by observing >30 glomeruli from each section. All slides were counterstained with hematoxylin. Normal goat IgG was used as a negative control.

Western blotting. Briefly, kidneys or liver was homogenized in radioimmunoprecipitation assay (RIPA) Lysis buffer (Santa Cruz Biotechnology) at 4°C. Similarly, cultured podocytes were lysed using RIPA buffer as previously described (17, 26). After centrifugation at 13,000 rpm for 30 min at 4°C, supernatant was collected and stored at −80°C until use. Total protein concentration was determined by using a DC-protein determination system (Bio-Rad Laboratories) using BSA as a standard. Samples were processed for SDS-PAGE, and proteins were electrotransferred onto nitrocellulose membranes with iBlot Dry Blotting System (Invitrogen). The membranes were blocked with 5% nonfat dry milk in 1 X TBS (0.1% Tween 20) for 1 h, incubated overnight with polyclonal rabbit anti-mouse TGFβ1/3 (Santa Cruz Biotechnology), polyclonal rabbit anti-VEGF-A, anti-VEGFR-2 (Santa Cruz Biotechnology), hamster anti-mouse MCP-1 (BioLegend, San Diego, CA), monoclonal anti-human VASH-1 (45), or polyclonal rabbit anti-mouse VASH-1 (37) antibodies at 4°C. After incubation with HRP-labeled secondary antibodies for 1 h, signals were detected with the ECL system (Amersham). Membranes were reprobed with rabbit polyclonal anti-actin antibodies (Bio-Rad) to serve as controls for equal loading. The density of each band was determined by using Image J software and expressed as a value relative to the density of a corresponding band obtained from actin immunoblot.

Recombinant VASH-1. Recombinant human VASH-1 was prepared as previously described (45). Human VASH-1 protein connected to the FLAG tag at the C terminus was expressed in a Bac-to-Bac baculovirus expression system (Life Technologies, Tokyo, Japan) according to the manufacturer’s instructions and purified as a soluble protein (45).

Cell culture. Conditionally immortalized mouse podocytes, generous gifts from Prof. Peter Mundel (University of Miami Miller School of Medicine, Miami, FL), were cultured on type I collagen-coated flasks (BD Falcon, San Jose, CA) with RPMI 1640 (Invitrogen) containing 10% FCS (Cansaer International), 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). To propagate podocytes, cells were cultivated at 33°C and treated with 50 U/ml of recombinant mouse IFN-γ (BD Bioscience, Palo Alto, CA) to enhance expression of the large T antigen. After 90% confluence, the cells were induced to differentiate into podocyte lineage by shifting them to 37°C and culturing in DMEM (Sigma) that contained 10% FCS without IFN-γ.

### Table 1. Body weight, blood glucose concentration, and food consumption

<table>
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<tr>
<th>Week 0</th>
<th>Week 8</th>
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<tbody>
<tr>
<td>Nondiabetic</td>
<td>Blood Glucose, mmol/l</td>
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<tr>
<td>Nondiabetic</td>
<td>26.7 ± 0.3</td>
</tr>
<tr>
<td>Diabetic (vehicle)</td>
<td>39.4 ± 0.2*</td>
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<tr>
<td>Diabetic (Ad-LacZ)</td>
<td>39.8 ± 0.5*</td>
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<tr>
<td>Diabetic (Ad-VASH-1)</td>
<td>40.0 ± 0.3*</td>
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Values are means ± SE; n = 6/group. Vehicle; vehicle buffer-treated; VASH-1, vasohibin-1. *P < 0.01 vs. nondiabetic controls.

### Table 2. Mean serum insulin, liver weight, heart weight, and right kidney weight in experimental animals

<table>
<thead>
<tr>
<th>Week 8</th>
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<tr>
<td>Group</td>
</tr>
<tr>
<td>Nondiabetic</td>
</tr>
<tr>
<td>Diabetic (vehicle)</td>
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<tr>
<td>Diabetic (Ad-LacZ)</td>
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<td>Diabetic (Ad-VASH-1)</td>
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Values are means ± SE; n = 6/group. *P < 0.05 vs. nondiabetic controls. †P < 0.05 vs. diabetic (vehicle) or diabetic (Ad-LacZ).
i.e., nonpermissive conditions. Under this condition, the majority of cells had an arborized shape and expressed podocyte-specific synaptopodin. After 7 days of culture under nonpermissive conditions, the cells were further cultured in DMEM that contained 0.5% FCS for 24 h, and quiescent mature podocytes were incubated with 5.5 mM normal glucose with PBS (NG), NG with 19.5 mM mannitol (NG/Manni), 25 mM high glucose with PBS (HG/V0), HG with 1 nM rhVASH-1 (HG/V1), 10 nM rhVASH-1 (HG/V10), or 20 nM rhVASH-1 (HG/V20) for 24 h. They then were harvested and subjected to Western blot analysis to determine the direct effect of rhVASH-1 on the protein levels of VEGF-A, ZO-1, P-cadherin (R&D Systems, Minneapolis, MN), desmin, FSP-1 (Dako Cytomation), Snail (Abcam), and Slug (Abcam). In other sets of experiments, cells were subjected to immunofluorescent staining for ZO-1 and P-cadherin.

Podocytes were also cultured in the presence of 1, 25(OH)2D3 (10 nM, Chugai Pharmaceutical, Tokyo, Japan) and all-trans-retinoic acid (ATRA; 1 μM, Sigma) to induce the expression of nephrin as previously reported (40).

**Immunofluorescent studies (cultured podocytes).** Mouse podocytes were cultured on eight-well chamber slides precoated with type I collagen (Nunc-Immuno Plate) and then fixed with ice-cold acetone for 5 min. The chamber slides were blocked with Protein Block Serum-Free (Dako Cytomation) and then incubated with primary antibodies, anti-ZO-1, or P-cadherin antibodies for 1 h. Subsequently, the slides were washed three times in PBS and incubated with Alexa Fluor 488-labeled donkey anti-rabbit IgG (A21206; Invitrogen) or Alexa Fluor 546-labeled goat anti-rat IgG (A11081; Invitrogen) for 1 h. After three washes with PBS, Vectashield anti-fade mounting medium (Vector Labs) was applied, the slides were observed by a fluorescence microscope (BZ-Analyzer; Keyence, Osaka, Japan), and images were obtained. Normal rat and hamster IgG were used as negative controls. Cells were double-stained with 4', 6-diamidino-2-phenylindole HCl to visualize nuclei.

**Fig. 2.** A–E: immunofluorescent staining of CD31, an endothelial cell marker. Distribution of CD31 was determined by indirect immunofluorescence technique in db/m mice (A), db/db mice treated either with vehicle buffer (B), AdLacZ (C), or AdhVASH-1 (D) at 16 wk of age. E: glomerular CD31+ endothelial area was quantitated. Increase in the CD31+ glomerular capillary area was significantly suppressed after treatment with AdhVASH-1. *P < 0.05 vs. db/m. †P < 0.05 vs. Ve or LacZ. F and G: immunoblot analysis. Immunoblots for VEGF-A, VEGF receptor (VEGFR)-2, and actin are shown. Each lane was loaded with 50 μg protein obtained from the renal cortex. Each band was scanned and subjected to densitometry. F, bottom: intensities of VEGF-A protein relative to actin. *P < 0.05 vs. db/m. †P < 0.05 vs. Ve or LacZ. G, bottom: intensities of VEGFR-2 protein relative to actin. *P < 0.05 vs. db/m. †P < 0.05 vs. Ve or LacZ; n = 6/group.
RNA extraction and quantitative real-time RT-PCR. Cultured mouse podocytes were homogenized and total RNA was extracted using an RNasy Midi Kit (Qiagen, Chatsworth, CA) and stored at −80°C until use. Total RNA was subjected to RT using a first-strand cDNA synthesis system (Invitrogen) with random hexamers and reverse transcriptase. Quantitative real-time PCR was used to quantify the mRNA levels of nephrin and the amount of 18S rRNA. cDNA was diluted 1:10 with autoclaved deionized water. For the detection of nephrin mRNA levels, 5 μl of the diluted cDNA was added to the Lightcycler-Mastermix, 0.5 μM of specific primer, 3 mM of MgCl₂ and 2 μl of Master SYBR Green. For detecting the level of 18S rRNA, 5 μl of the diluted cDNA was added to the Lightcycler-Mastermix, 0.2 μM of specific primer, 3 mM of MgCl₂ and 2 μl of SYBR Premix Ex Taq (Takara Bio). These reaction mixtures were filled to a final volume of 20 μl with water. PCR reactions were carried out in a real-time PCR cycler (Lightcycler; Roche Diagnostics). The program was optimized and performed finally as denaturation at 95°C for 10 min followed by 40 cycles of amplification (nephrin; 95°C for 10 s; 60°C for 20 s, 18S rRNA; 95°C for 5 s; 60°C for 20 s, respectively). The temperature ramp rate was 20°C/s. At the end of each extension step, the fluorescence was measured to quantitate the PCR products. After completion of the PCR, the melting curve of the product was measured by a temperature gradient from 65 to 95°C at 0.1 or 0.2°C/s with 0.05 was considered statistically significant.

RESULTS

Serum and hepatic levels of VASH-1 following adenoviral transfer. The serum and hepatic levels of VASH-1 were detected by immunoblot assay. Serum levels of VASH-1 were at very low levels in both the nondiabetic db/m and the control diabetic db/db mice (data not shown). The AdhVASH-1-injected diabetic db/db mice exhibited significantly elevated serum VASH-1 levels compared with the AdLacZ injection at 2 wk after the final injections (Fig. 1A). Similarly, hepatic expression of VASH-1 was markedly elevated in the AdhVASH-1-injected db/db mice (Fig. 1A). The mice receiving AdLacZ or AdhVASH-1 did not exhibit any deleterious side effects, and all the mice survived. In addition, nondiabetic male db/m mice receiving AdhVASH-1 did not exhibit any inflammatory or pathological alterations in the lungs, liver, or kidneys (data not shown), and hypertension or proteinuria was not observed.

Fig. 3. A–D: glomerular accumulation of type IV collagen was assessed by the indirect immunofluorescence method for db/m mice (A), db/db mice treated either with vehicle buffer (B), AdLacZ (C), or Adh-VASH-1 (D) at 16 wk of age. Original magnification, ×400. Immunohistochemistry of F4/80⁺ monocytes/macrophages. Representative light microscopic appearances of glomerulus in db/m mice (E), db/db mice treated with either vehicle buffer (F), AdLacZ, (G), or AdhVASH-1 (H) are shown. F4/80⁺ cells were observed in glomeruli of db/db mice (arrowheads; original magnification ×400). I: amount of immunoreactive type IV collagen in glomeruli relative to the nondiabetic control db/m mice determined by computer image analysis is shown. J: number of glomerular F4/80⁺ monocyte/macrophages is shown. Increase in the number of F4/80⁺ monocyte/macrophages was significantly suppressed after treatment with AdhVASH-1; n = 6/group. *P < 0.05 vs. N. †P < 0.05 vs. Ve or LacZ.
Changes in blood glucose, body weight, food consumption, mean serum insulin, liver weight, heart weight, and kidney weight. Body weight, blood glucose, food consumption, serum levels of insulin, liver weight, heart weight, and kidney weight were significantly increased in the db/db mice compared with the db/m mice at the age of 16 wk. Treatment with AdhVASH-1 did not alter plasma glucose concentrations, food consumption, obesity, and mean serum insulin in db/db mice after 8 wk of treatment (Tables 1 and 2). There was no significant difference in liver weight between treatment with AdhVASH-1 and AdLacZ, but the increase in kidney weight in db/db mice was significantly suppressed by treatment with AdhVASH-1 compared with AdLacZ (Table 2). Heart weight tended to be lower in the AdhVASH-1-treated group compared with the AdLacZ-treated group without statistical significance (Table 2).

Changes in blood pressure. There were no significant differences in blood pressure measured at the age of 16 wk among the experimental groups (db/m mice, 118 ± 5; db/db mice treated with vehicle, 114 ± 3; db/db mice treated with AdLacZ, 114 ± 3; db/db mice treated with AdhVASH-1, 112 ± 5, mmHg).

Changes in serum creatinine, Ccr, and urinary albumin excretion. Serum creatinine levels did not significantly differ among the experimental groups. To evaluate the effect of VASH-1 on preventing hyperfiltration associated with diabetic nephropathy, Ccr and UACR were determined (Fig. 1, B and C). Although db/db mice treated with vehicle or AdLacZ showed a marked elevation of Ccr and UACR, AdhVASH-1 treatment resulted in the suppression of the diabetes-induced increase in Ccr and UACR at 8 wk after initiation of treatment.

Histology and morphometric analysis. Histological examination of the kidneys revealed glomerular hypertrophy and expansion of the mesangial area in vehicle-treated db/db mice. At 8 wk after initiation of treatment with AdhVASH-1, glomerular hypertrophy and mesangial matrix expansion were significantly inhibited compared with the control diabetic animals (Fig. 1, D–G). Morphometric analysis (Fig. 1, H and I) further confirmed the inhibitory effects of AdhVASH-1 on these parameters.

Immunohistochemical analysis of CD31+ endothelial area. We next evaluated differences in the CD31+ glomerular endothelial area by immunofluorescence staining. In db/m mice, immunoreactivity for CD31 was detected in glomerular capillaries (Fig. 2A), and an increase in the CD31+ area in glomeruli was observed in the control diabetic db/db mice (Fig. 2, B and C). Treatment with AdhVASH-1 significantly suppressed the increase in the glomerular CD31+ area compared with AdLacZ (Fig. 2D), confirmed by quantitative analysis (Fig. 2E). We also evaluated the CD31+ PTC endothelial area to determine the potential adverse effects of VASH-1 on the survival of PTC at 8 wk after initiating treatment. No significant difference of PTC density was observed among experimental groups (Supplementary Fig. S1; all supplementary material for this article is available on the journal web site).

Protein levels of VEGF-A and receptor VEGFR-2 in the renal cortex. The effect of AdhVASH-1 on the expression of proangiogenic factor VEGF-A and corresponding receptors VEGFR-2 in the renal cortex was studied by immunoblot assay. The level of VEGF-A and VEGFR-2 was significantly increased in db/db mice, which is consistent with the findings of previous reports (7, 17, 18, 29), and treatment with AdhVASH-1 significantly suppressed the increase in VEGF-A and VEGFR-2 compared with AdLacZ in db/db mice (Fig. 2, F and G).

Immunohistochemical analysis of glomerular type IV collagen. Next, the accumulation of glomerular type IV collagen was examined by immunofluorescence staining (Fig. 3). The amount of type IV collagen in glomeruli was increased in the control db/db mice (Fig. 3, B and C) compared with nondiabetic db/m mice (Fig. 3A). Enhanced immunoreactivity in the diabetic mice was observed mainly in the glomerular basement membrane and mesangial area. Treatment with AdhVASH-1 decreased the accumulation of type IV collagen compared with AdLacZ treatment in db/db mice (Fig. 3D), and these results were further confirmed by quantitative morphometric analysis (Fig. 3I).

Immunohistochemical analysis of monocyte/macrophage accumulation. We next examined the glomerular infiltration of monocytes/macrophages by immunohistochemistry for F4/80.
In the vehicle-treated \(db/db\) mice (Fig. 3F), the number of F4/80\(^+\) cells in glomeruli was significantly increased compared with the nondiabetic \(db/m\) mice (Fig. 3E). Treatment with AdhVASH-1 markedly decreased the glomerular accumulation of monocytes/macrophages compared with AdLacZ treatment (Fig. 3, G, H, and J).

Protein levels of TGF-\(\beta_1\) and MCP-1 in the renal cortex. TGF-\(\beta_1\) plays crucial roles in mesangial matrix expansion and renal hypertrophy in diabetic nephropathy (35). MCP-1 is a crucial chemokine involved in the development of diabetic nephropathy (42). The control diabetic \(db/db\) mice exhibited increased protein levels of TGF-\(\beta\) and MCP-1 compared with
the nondiabetic db/m mice in the renal cortex, as detected by immunoblotting. AdhVASH-1 significantly suppressed the increase in TGF-β and MCP-1 compared with AdLacZ in the diabetic animals (Fig. 4, A and B).

**Immunohistochemical analysis of podocyte injuries and slit diaphragm proteins.** Since we observed antialbuminuric effects of VASH-1, we next evaluated the degree of podocyte injuries among the experimental groups by immunohistochemistry for nephrin, ZO-1, desmin, and FSP-1. In the nondiabetic db/m mice, localization of nephrin (Fig. 5A) and ZO-1 (Fig. 5E) was observed along the glomerular capillary wall in a continuous pattern, suggesting the localization in podocytes. In contrast, immunoreactivity for desmin, a marker for mesenchymal phenotype and podocyte injuries, was not observed in the nondiabetic db/m mice (Fig. 5I). Similarly, immunoreactivity for FSP-1, a marker of EMT, was not observed in the glomeruli in nondiabetic db/m mice (Fig. 5M). In the control diabetic db/db mice, the intensity of nephrin and ZO-1 staining was diminished and observed as a discontinuous pattern along the glomerular capillary walls (Fig. 5, B, C, F, and G), and immunoreactivity for desmin and FSP-1 was observed along the periphery of glomerular capillaries (Fig. 5, J, K, N, and O), suggesting podocyte injuries. Treatment with AdhVASH-1 restored the protein levels as well as the continuous patterns for nephrin and ZO-1 and suppressed the number of desmin⁺ and FSP-1⁺ cells in db/db mice (Fig. 5, D, H, L, and P).

**Protein levels of VEGF-A in cultured mouse podocytes.** We next performed cell culture analysis using mouse podocytes to examine the potential direct effects of VASH-1 on podocytes in association with its antialbuminuric effects. The protein level of VEGF-A in podocytes was significantly increased at 24 h under the HG condition compared with the NG condition as detected by immunoblotting (Fig. 6). Addition of mannitol to the NG condition did not lead to the increase in VEGF-A, thus excluding the potential effect by elevated osmotic pressure. Treatment with rhVASH-1 resulted in the suppression of the increase in VEGF-A protein induced by HG in a dose-dependent manner (Fig. 6).

**Protein levels of ZO-1, P-cadherin, desmin, and FSP-1 in cultured mouse podocytes.** The effects of rhVASH-1 on the expression of ZO-1 and P-cadherin (epithelial cell proteins) or desmin and FSP-1 (mesenchymal cell markers) in podocytes were studied by immunoblotting. The levels of P-cadherin and ZO-1 in podocytes were significantly decreased at 24 h under HG compared with NG. In contrast, the protein levels of desmin and FSP-1 were significantly increased at 24 h under HG. Treatment with rhVASH-1 significantly recovered the HG-induced loss of P-cadherin and ZO-1 protein (Fig. 7, A–C) and suppressed the HG-induced increase in protein levels of desmin and FSP-1 (Fig. 7, D–F).

**Protein levels of Snail and Slug in cultured mouse podocytes.** We next examined the expression of Snail and Slug, the transcription factors involved in EMT, in podocytes. The protein levels of Snail and Slug were increased at 24 h after incubating under HG compared with NG as detected by immunoblotting. Treatment with rhVASH-1 significantly suppressed the increase in the protein levels of Snail and Slug induced by HG (Fig. 8, A and B).

**Immunohistochemical analysis of ZO-1 and P-cadherin in cultured mouse podocytes.** Immunofluorescence staining showed that ZO-1 and P-cadherin were localized in the cell-to-cell junctions of podocytes under NG, and immunoreactivity for these proteins was diminished under HG. Treatment with rhVASH-1 markedly recovered the immunoreactivity for ZO-1 and P-cadherin in the cell-to-cell junctions of podocytes (Fig. 9).

**mRNA levels of nephrin in cultured mouse podocytes.** Since the expression of nephrin is hardly detectable in these cultured mouse podocytes, we induced the expression of nephrin in the presence of 1, 25(OH)₂D₃ and all-trans-retinoic acid (VRAD)
as previously described (40). Sufficient induction of nephrin mRNA was confirmed as detected by real-time PCR in podocytes under VRAD and NG, but nephrin mRNA levels were significantly reduced under VRAD and HG (Fig. 10). Treatment with rhVASH-1 significantly recovered the HG-induced loss of nephrin mRNA (Fig. 10).

Levels of endogenous mouse VASH-1 in nondiabetic and diabetic mouse kidneys. We next examined the levels of endogenous mouse VASH-1 protein levels in the kidneys of the experimental groups as detected by Western blotting. Renal levels of mouse VASH-1 were increased in the control diabetic mice compared with nondiabetic mice, suggesting the compensatory increase in VASH-1 in response to an excessive angiogenic milieu with elevation of VEGF-A. Treatment with AdhVASH-1 did not alter the levels of endogenous mouse VASH-1 in kidney. Based on these findings, we speculate that the increase in mouse VASH-1 in the diabetic kidney may not be sufficient to counteract the effects of angiogenic stimuli such as VEGF-A, requiring the supplementation of exogenous VASH-1 to effectively suppress the progression of diabetic nephropathy.

Intravenous administration of AdhVASH-1 resulted in the sustained increase in serum levels of hVASH-1 presumably derived from the liver, without causing any systemic inflammation. Additionally, administration of AdhVASH-1 in nondiabetic mice did not cause any renal inflammatory alterations (data not shown). Therefore, we consider the present approach...
employing adenoviral vectors to be nonharmful for experimental animals. AdhVASH-1 treatment did not affect blood glucose, body weight, food consumption, or serum insulin concentration in db/db mice. Previous reports have described inconsistent results on Ccr within 2 mo of the onset of diabetes in db/db mice (6, 12). The discrepant outcomes may be attributed to a variable susceptibility to diabetes in sub培育ings of the db/db mouse strain. In the present study, we observed increased Ccr and albuminuria in vehicle-treated db/db mice. In addition, characteristic histological alterations in diabetic nephropathy such as glomerular hypertrophy and the increase in mesangial matrix were observed in the control diabetic mice. These abnormalities in diabetic nephropathy were significantly inhibited by AdhVASH-1 compared with AdLacZ treatment at 8 wk after initiation of treatment. The suppressive effects of AdhVASH-1 on albuminuria were rather modest compared with the suppressive effects on the increase in Ccr, suggesting the requirement of additional factors or the involvement of distinct mechanisms in albuminuria in diabetic nephropathy.

Experimental type 1 and type 2 diabetes animals exhibit an increased glomerular filtration surface area, glomerular capillary length, and/or glomerular capillary number (14, 30). Treatment with AdhVASH-1 suppressed the increase in the CD31+ glomerular endothelial area in db/db mice, potentially via its antiangiogenic efficacy, thus leading to the observed suppressive effects on the increase in Ccr and albuminuria. Previous studies demonstrated the therapeutic effects of neutralizing anti-VEGF-A antibodies in diabetic nephropathy models (8, 12) and amelioration of diabetic nephropathy in mice with inducible overexpression of sFlt-1 in podocytes (21). In the present study, we observed increased renal levels of VEGF-A in the diabetic control mice, consistent with the findings of previous reports (7, 29, 41, 48). On the contrary, recent reports have demonstrated reduced expression of VEGF-A in human diabetic nephropathy (1, 23). Although diabetic animal models are often studied in a relatively early phase of the disease, most diabetic patients employed in clinical studies were already in a moderately advanced stage. In a study using samples of human diabetic nephropathy, reduced interstitial VEGF-A expression was associated with interstitial vascular rarefaction (23), but we detected no reduction of PTC density in the present study in the control db/db mice. Treatment with AdhVASH-1 suppressed the increase in VEGF-A as well as flk-1 in the renal cortex. In our previous analysis, AdhVASH-1 treatment failed to suppress renal levels of VEGF-A, but suppressed the levels of flk-1 in type 1 diabetic mice (29). The discrepant results regarding the regulation of renal VEGF-A levels by VASH-1 between type 1 and 2 diabetes models may be attributed to the difference in models of diabetes and stages of diabetic nephropathy. Since podocytes are the main source of VEGF-A in glomeruli, we then examined the potential direct effect of VASH-1 on the synthesis of VEGF-A induced by high glucose in podocytes. We observed the inhibitory effect of VASH-1 on high glucose-induced upregulation of VEGF-A in cultured mouse podocytes.

Renal hypertrophy observed in the control db/db mice was significantly suppressed by AdhVASH-1. VEGF-A augments protein synthesis and hypertrophy in renal proximal tubular epithelial cells (34). Considering the dominant contribution of the tubular compartment in organizing renal mass, VASH-1 might have regulated glomerular as well as tubular hypertrophy potentially via regulating VEGF-A-mediated signaling.

The accumulation of type IV collagen in glomeruli in db/db mice was also inhibited by AdhVASH-1, potentially associated with the suppression of renal TGF-β1 levels. These results are consistent with the inhibitory effects of other antiangiogenic reagents on ECM accumulation in animal models of diabetic nephropathy (17, 18, 44, 48, 50). We recently reported the inhibitory effects of recombinant VASH-1 on the high glucose-induced increase in TGF-β in cultured mesangial cells (29), suggesting the possibility of the direct effects of VASH-1 on mesangial cells leading to ameliorated accumulation of glomerular type IV collagen. In addition, podocyte-derived VEGF-A induced by TGF-β1 was reported to stimulate the production of α3(IV) collagen, a component of α-chains in GBM (5). Therefore,
the inhibitory effect of VASH-1 on mesangial matrix expansion may also be mediated through regulation of VEGF-A, which presumably is involved in mediating the profibrotic effect of TGF-β1 in diabetic nephropathy.

The potential role of VEGF-A in mediating glomerular monocyte/macrophage infiltration has been demonstrated in a diabetic animal model (33). The anti-inflammatory effect of VASH-1 observed in the present study might be associated with regulation of vascular permeability through suppressing overactivation of VEGF-A signaling and with inhibition of renal MCP-1 levels. In fact, the therapeutic effect of VASH-1 on the formation of arterial neointima in association with inhibitory effects on adventitial macrophage infiltration has been reported (49). Since infiltration of macrophages and mesangial accumulation of ECM proteins are associated in diabetic nephropathy (31, 32), the observed anti-inflammatory effects of VASH-1 might have contributed to the inhibition of mesangial matrix accumulation.

Podocyte injury in association with altered expression of slit diaphragm proteins is involved in proteinuria in diabetic nephropathy. In addition, recent reports suggested the involvement of EMT-like alterations in podocytes in association with the loss of glomerular filtration barrier function in diabetic nephropathy (22, 47). Since AdhVASH-1 showed inhibitory effects on albuminuria in db/db mice, we evaluated the expression of nephrin, ZO-1, and P-cadherin, important components of the slit diaphragm cell adhesion complexes (3). We ob-

Fig. 9. A–C: immunofluorescent staining of zonula occludens (ZO)-1. Distribution of ZO-1 (green) was determined by indirect immunofluorescence technique in cultured podocytes incubated with NG (A), HG without rhVASH-1 (B), or HG with 20 nM rhVASH-1 (C). D–F: immunofluorescent staining of P-cadherin. Distribution of P-cadherin (red) was determined by indirect immunofluorescence staining in cultured podocytes incubated with NG (D), HG without rhVASH-1 (E), or HG with 20 nM rhVASH-1 (F). Cells were double stained with 4',6-diamino-2-phenylindole HCL to visualize the nuclei. Original magnification ×400.

Fig. 10. Expression of nephrin mRNA detected by real-time PCR. Total RNA was extracted from cultured podocytes and subjected to quantitative real-time PCR as described in MATERIALS AND METHODS. The amount of nephrin mRNA relative to 18S rRNA is shown. Results were expressed relative to NG-VRAD(−) that were arbitrarily assigned a value of 1.0. *P < 0.01 vs. NG-VRAD(−), †P < 0.05 vs. NG-VRAD(−), ‡P < 0.01 vs. NG-VRAD(+) or NG/Manni-VRAD(+) or NG/Manni-VRAD(+). #P < 0.05 vs. HG/V0-VRAD(+) or HG/V1-VRAD(+). $P < 0.01 vs. HG/V0-VRAD(+) or HG/V1-VRAD(+); n = 6/group.

Fig. 11. Immunoblot analysis of mVASH-1. Immunoblots for mVASH-1 and actin are shown. Each lane was loaded with 50 μg protein obtained from the renal cortex. Bottom: intensities of mVASH-1 protein relative to actin. *P < 0.05 vs. db/m; n = 6/group.
served the reduction as well as altered localization of nephrin, P-cadherin, and ZO-1 in the control db/db mice, and AdhVASH-1 treatment significantly recovered the altered expression of these proteins. The inhibitory effects of AdhVASH-1 on the increase in desmin- podocytes and FSP-1- podocytes further confirmed the protective effects of VASH-1 on podocytes via suppressing EMT-like alterations. VASH-1 further maintained the levels of nephrin, epithelial cell-specific proteins (ZO-1 and P-cadherin) and reduced the levels of mesenchymal proteins (desmin and FSP-1) under HG in cultured podocytes. In addition, the HG-induced increase in the protein levels of transcription factors Snail and Slug, involved in the induction of EMT, was inhibited by VASH-1. Our results suggest the potential therapeutic effects of VASH-1 via counteracting EMT-like alterations and maintaining the characteristic phenotype of podocytes in diabetic nephropathy.

There are several limitations to the present study. In the advanced stage of diabetes, antiangiogenic reagents may impair neovessel formation, leading to deterioration of macrovascular complications such as myocardial infarction and limb ischemia. However, AdhVASH-1 might be tolerable or even therapeutic for atherosclerotic conditions considering the therapeutic effects of AdhVASH-1 in preventing neointimal formation (49). The crucial involvement of chronic hypoxia in association with a reduction of PTC in progressing tubulointerstitial injuries has been reported (28). AdhVASH-1 did not reduce PTC density in the present model, suggesting its safety in diabetic nephropathy. However, careful evaluation on the potential influence of VASH-1 on PTC density at a more advanced stage of diabetic nephropathy might be required.

Recently, the potential adverse events of bevacizumab, a humanized monoclonal antibody against VEGF-A, resulting in thrombotic microangiopathy have been reported in patients with cancer (10). The lack of such histological alterations in previous experimental diabetic nephropathy models employing anti-VEGF-A antibodies or SU5416 (8, 12, 38) as well as amelioration of diabetic glomerular alterations by inducible podocyte-specific overexpression of sFlt-1 in adult mice (21) suggest that anti-VEGF-A therapy might not be detrimental for patients with diabetic nephropathy. In fact, systemic delivery of sFlt-1 resulted in the protection of podocytes associated with reduction of albuminuria, but led to the exacerbation of tubulointerstitial injuries accompanied by peritubular capillary loss in db/db mice (20). Although VASH-1 suppresses overactivation of VEGFR-2 (29), it does not serve as a specific inhibitor of VEGF signaling (45), does not cause apoptosis of normal vascular endothelial cells (16), and did not cause peritubular capillary loss or tubulointerstitial injuries in the present study. In addition, AdhVASH-1 did not cause proteinuria in nondiabetic mice. Thus VASH-1 treatment is distinct from strategies targeting VEGF-A, potentially associated with fewer adverse events. The identification of specific receptors for VASH-1 is underway. Alteration in the expression of VASH-1 in the normal kidney and in patients with renal disorders is also under investigation.

In conclusion, we demonstrated that VASH-1 effectively ameliorates renal alterations in an animal model of type 2 diabetes. Our results implicate the direct effects of VASH-1 on glomerular podocytes for the first time in association with anti-proteinuric mechanisms via maintaining the epithelial phenotype. We believe that our present study will eventually guide us to the development of novel therapeutic strategies for patients with diabetic nephropathy.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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