Vasohibin-1, a Negative Feedback Regulator of Angiogenesis, Ameliorates Renal Alterations in a Mouse Model of Diabetic Nephropathy

Tatsuyo Nasu,[1] Yohei Maeshima,[1] Masaru Kinomura,[1] Kumiko Hirokoshi-Kawahara,[1] Katsuyuki Tanabe,[1] Hitoshi Sugiyama,[1,2] Hikaru Sonoda,[3] Yasufumi Sato,[4] and Hirofumi Makino[1]

OBJECTIVE—The involvement of proangiogenic factors such as vascular endothelial growth factor as well as the therapeutic efficacy of angiogenesis inhibitors in early diabetic nephropathy has been reported. Vasohibin-1 (VASH-1) is a unique endogenous angiogenesis inhibitor that is induced in endothelial cells by proangiogenic factors. We investigated the therapeutic efficacy of VASH-1 in an early diabetic nephropathy model.

RESEARCH DESIGN AND METHODS—Streptozotocin-induced type 1 diabetic mice received intravenous injections of adenoviral vectors encoding VASH-1 (AdhVASH-1) or β-gal (Ad-LacZ) every other week and were killed after 28 days.

RESULTS—Treatment with AdhVASH-1 resulted in sustained increase in the protein levels of VASH-1 in the liver and sera, in the absence of any inflammatory alterations. AdhVASH-1 treatment significantly suppressed renal hypertrophy, glomerular hypertrophy, glomerular hyperfiltration, albuminuria, increase of the $CD31^+$ glomerular endothelial area, $F4/80^+$ monocyte/macrophage infiltration, the accumulation of type IV collagen, and mesangial matrix compared with AdLacZ-treated diabetic mice. Increase in the renal levels of transforming growth factor-β1, monocyte chemoattractant protein-1, and receptor for advanced glycation end products in diabetic animals was significantly suppressed by AdhVASH-1 (real-time PCR and immunoblot). VASH-1 significantly suppressed the increase of transforming growth factor-β, monocyte chemoattractant protein-1, and receptor for advanced glycation end products, induced by high ambient glucose in cultured mouse mesangial cells. Increased phosphorylation of VEGFR2 was suppressed in AdVASH-1–treated diabetic animals and in cultured glomerular endothelial cells. Endogenous mouse VASH-1 was localized to the mesangial and endothelial area in glomeruli of diabetic mice.

CONCLUSIONS—These results suggest the potential therapeutic efficacy of VASH-1 in treating early diabetic nephropathy potentially mediated via glomerular endothelial and mesangial cells. ***Diabetes*** **58:2365–2375, 2009**

From the [1]Department of Medicine and Clinical Science, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan; the [2]Center for Chronic Kidney Disease and Peritoneal Dialysis, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan; the [3]Discovery Research Laboratories, Shionogi, Osaka, Japan; and the [4]Department of Vascular Biology, Institute of Development, Aging, and Cancer, Tohoku University, Sendai, Japan.

Corresponding author: Yohei Maeshima, ymaeshim@md.okayama-u.ac.jp.

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Diabetic nephropathy is a major microvascular complication of type 1 and 2 diabetes, and 30–40% of patients with type 2 diabetes develop diabetic nephropathy. Because diabetic nephropathy is the most common pathological disorder predisposing end-stage renal disease (ESRD) in Japan and in the Western world, novel therapeutic approaches are required. In the early stage of diabetic nephropathy, glomerular hyperfiltration, glomerular and tubular epithelial hypertrophy, microalbuminuria, and thickening of the glomerular basement membrane are typically observed. Expansion of the extracellular matrix (ECM) in mesangial areas and overt proteinuria are observed, eventually leading to glomerulosclerosis and ESRD (1). The involvement of various factors and cytokines including the renin-angiotensin system, IGF-I, monocyte chemoattractant protein-1 (MCP-1), fibrogenic transforming growth factor-β1 (TGF-β1), protein kinase C (PKC), and advanced glycation end products (AGE) in diabetic nephropathy has been reported (2,3).

Angiogenesis is associated with pathological conditions including tumor growth and diabetic retinopathy (4). Vascular endothelial growth factor (VEGF)-A, a potent stimulator of angiogenesis, promotes endothelial cell proliferation, migration, and tube formation (5), and induces vascular permeability and inflammation (6).

Previous studies have demonstrated the increased glomerular filtration surface in diabetic nephropathy resulting from the formation of new glomerular capillaries and a slight elongation of the preexisting capillaries (7,8), analogous to the changes in pathological diabetic retinopathy.

The increase in the levels of VEGF-A and the receptor of VEGF-A, VEGFR2, has been reported in diabetic nephropathy models (9,10). In addition, the therapeutic efficacies of anti–VEGF-A strategies (i.e., neutralizing antibodies and a receptor tyrosine kinase inhibitor) have further demonstrated the involvement of VEGF-A in the progression of diabetic nephropathy (11–13).

The therapeutic effects of antiangiogenic reagents, tumstatin peptide, endostatin peptide, angiostatin, pigment epithelium–derived factor, and 2-(8-hydroxy-6-methoxy-1-oxo-1h-2-benzopyran-3-yl) propionic acid (NM-3) (14–18) 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 to investigate genes upregulated by VEGF in endothelial cells (19). The therapeutic effects of VASH-1 on tumor growth, atherosclerosis, and proliferative retinopathy models have been reported (19–21). Based on the unique characteris-

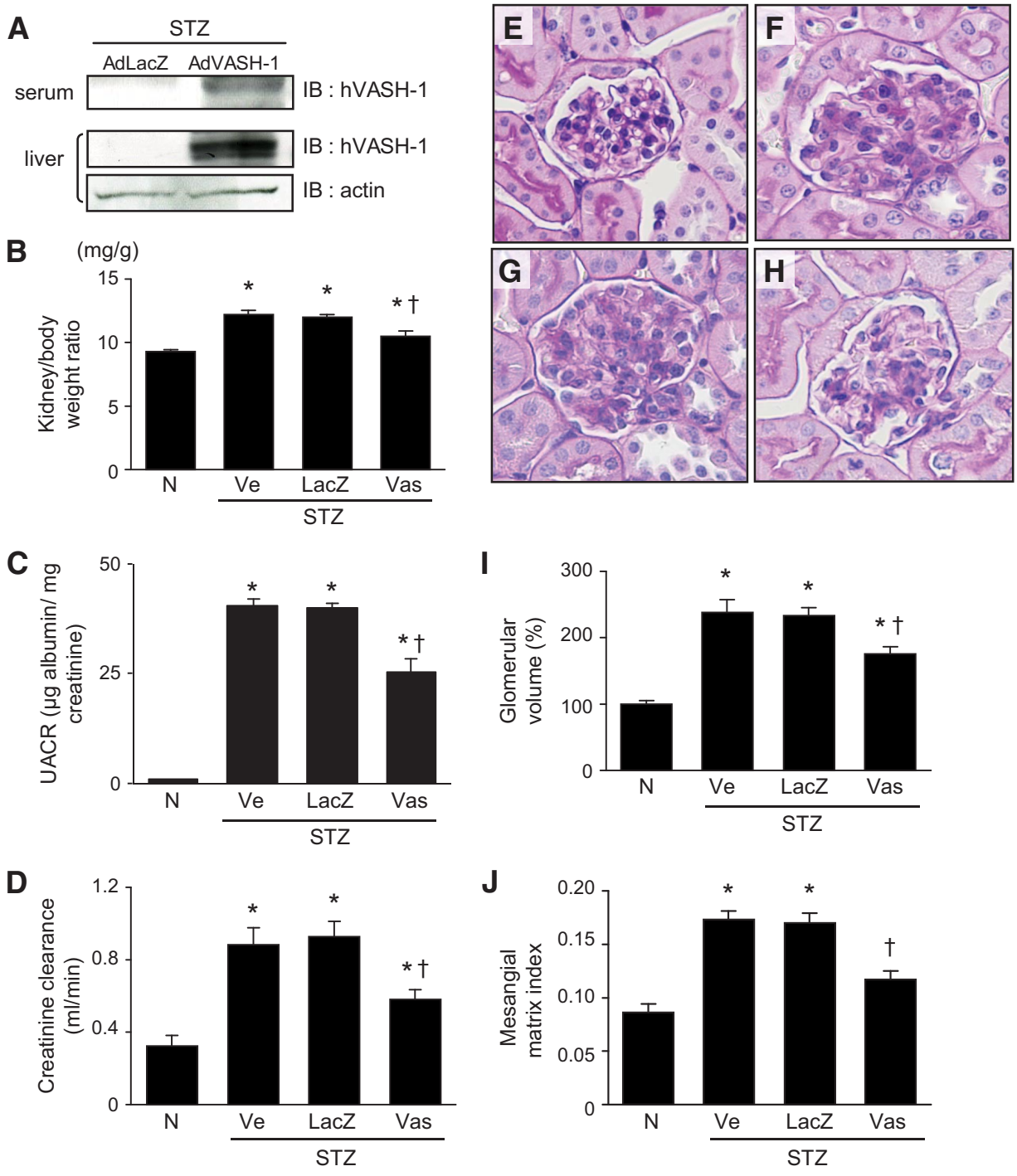

FIG. 1. *A*: Immunoblot analysis. Immunoblot for human VASH-1 and actin are shown. Each lane was loaded with 50 μg protein obtained from the serum samples or liver. The AdhVASH-1–injected diabetic mice exhibited significantly elevated serum VASH-1 (42 kDa) levels compared with the AdLacZ-injected diabetic mice (4 weeks). Similarly, enhanced protein levels of VASH-1 in the liver were observed in AdhVASH-1–treated mice compared with AdLacZ-treated diabetic mice. Immunoblots for actin are shown to confirm equal loading. *B*: Increase in KW-to-BW ratio induced by STZ was diminished in the AdhVASH-1–treated diabetic group. Kidney weight relative to body weight was determined before termination of the experiments. *C*: Increase in UACR induced by STZ was significantly suppressed by treatment with AdhVASH-1. Data obtained at 4 weeks after initiating treatment with Ad-LacZ or AdhVASH-1 is shown. *D*: Increase in Ccr induced by STZ was partially suppressed by AdhVASH-1. *B–D*: *$P < 0.05$ versus N. †$P < 0.05$ versus Ve or LacZ. *E–H*: Representative light microscopic appearance of glomeruli (periodic acid-Schiff staining, original magnification ×400) for nondiabetic control mice (*E*) and diabetic mice treated with either vehicle buffer (*F*), AdLacZ (*G*), or AdhVASH-1 (*H*). *I*: Increase in glomerular volume induced by STZ was diminished by treatment with AdhVASH-1. *J*: Mesangial matrix index was defined as the proportion of the glomerular tuft occupied by the mesangial matrix area (excluding nuclei). *I* and *J*: *$P < 0.01$ versus N. †$P < 0.01$ versus Ve or LacZ; $n = 5$ for each group. N, nondiabetic control; Ve, diabetic mice treated with vehicle buffer; LacZ, diabetic mice treated with AdLacZ (5×10^9 vp/mice); Vas, diabetic mice treated with AdhVASH-1 (5×10^9 vp/mice). Each column consists of means ± SE. (A high-quality digital representation of this figure is available in the online issue.)

tics of this factor, VASH-1 is considered to act as an endothelial cell–derived negative feedback regulator of angiogenesis.

In the present study, we demonstrate the therapeutic efficacy of VASH-1 in ameliorating renal alterations in the streptozotocin (STZ)-induced mouse type 1 diabetes model. Treatment with adenoviral vector encoding human VASH-1 (AdhVASH-1) markedly suppressed characteristic alterations of early diabetic nephropathy. These effects were associated with the regulation of VEGFR2 activation in glomerular endothelial cells and of TGF-β1, MCP-1, and receptor for advanced glycosylation end product (RAGE) in mesangial cells.

RESEARCH DESIGN AND METHODS

Adenoviral vectors. A replication-defective AdhVASH-1 was prepared as previously described (19). A replication-defective adenovirus vector encoding the *Escherichia coli* β-galactosidase (AdLacZ), which is identical to AdhVASH-1 except for the inserted cDNA, was used as the control (19) (see the online appendix available at http://diabetes.diabetesjournals.org/content/early/2009/07/08/db08-1790/suppl/DC1).

Induction of diabetes and experimental protocols. The experimental protocol was approved by the Animal Ethics Review Committee of Okayama University. Male imprinting control region mice were fed a standard pellet laboratory diet and were provided with water ad libitum. Type 1 diabetes was induced by low-dose STZ injection as detailed by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) Consortium for Animal Models of Diabetic Complications' (AMDCC) protocol (available from http://www.amdcc.org) with modification. Weight-matched 5-week-old male mice received intraperitoneal injections of STZ (Sigma, St. Louis, MO; 120 mg/kg body weight) dissolved in 10 mmol/l Sodium citrate, pH 5.5. Control mice received injections with buffer alone. STZ or citrate buffer was administered at three time points occurring at 48-h intervals during the first week. Six days after the third injection of STZ, mice with blood glucose in the range of 13.9–22.2 mmol/l were divided into four subgroups: *1*) nondiabetic control and diabetic mice treated with either, *2*) vehicle buffer (saline), *3*) AdLacZ, or *4*) AdhVASH-1 ($n = 5$ for each subgroup). Thirty-two mice received injections of STZ, and 15 mice exhibiting hyperglycemia in the range as described above were selected for experiments. At this point, intravenous injections of adenoviral vectors (AdLacZ or AdhVASH-1) or saline (via tail veins) were initiated using a syringe with a 27-gauge needle and were repeated every other week (5×10^9 vp/100 μl). Four weeks after the initial injections of adenoviral vectors (6 weeks after the induction of diabetes), mice were killed and the kidneys were obtained. No mice died and no signs of apparent exhaustion were observed during the experimental period (available in an online appendix).

Blood and urine examination. Blood glucose was measured in tail-vein blood, and urine was tested for keton bodies and glucose by the OML (Okayama Medical Laboratories, Okayama, Japan). Serum and urinary creatinine levels and urinary albumin concentration were determined as previously described (14). Results were normalized to the urinary creatinine levels and expressed as the urinary albumin-to-creatinine ratio (UACR). The creatinine clearance (Ccr) was calculated and expressed as milliliters per minute per 100 g of body weight (available in an online appendix).

Histological analysis. Four weeks after starting treatment, kidneys were removed, fixed in 10% buffered formalin, and embedded in paraffin. Sections (3 μm) were stained with periodic acid-Schiff for light microscopic observation (available in an online appendix).

Immunohistochemistry. Immunohistochemistry was performed using frozen sections as previously described (18,22–24) (available in an online appendix).

RNA extraction and quantitative real-time PCR. RNA extraction and real-time PCR were performed as previously described with modifications (14,18) (available in an online appendix).

Immunoblot assay. Immunoblot assay was performed as previously described (18,25) (available in an online appendix).

Recombinant VASH-1. Recombinant VASH-1 was prepared as previously described (19). Human VASH-1 protein connected to the FLAG tag at the C-terminus was expressed in a Bac-to-Bac baculovirus expression system (Invitrogen) according to the manufacturer's instructions and purified as a soluble protein (19).

Cell culture. Primary murine mesangial cells (MES13) were utilized to determine the direct effect of recombinant human VASH-1 on high glucose–induced increase of the protein levels of TGF-β, MCP-1, and RAGE as previously described (18,26) (available in an online appendix). Primary human glomerular microvascular endothelial cells (hGECs) were utilized to determine the direct effect of recombinant human VASH-1 on VEGF (R&D Systems) or high glucose–induced increase of the phosphorylation of VEGFR2 (available in an online appendix).

Statistical analysis. All values are expressed as means ± SE. A Kruskal-Wallis with post hoc comparisons using the Scheffe's test was employed for inter-group comparisons of multiple variables. Statistical analysis was performed by SPSS Software for Windows (version 13.0; Chicago, IL). A level of $P < 0.05$ was considered statistically significant.

TABLE 1
Body weight, blood glucose concentration, and blood pressure

	Body weight (g)	Blood glucose (mmol/l)	MBP (mmHg)
Nondiabetic	35.8 ± 0.2	7.3 ± 0.3	80.6 ± 3.0
Diabetic (vehicle)	30.8 ± 1.8*	27.3 ± 0.7*	80.9 ± 1.7
Diabetic (AdLacZ)	31.6 ± 2.1*	27.6 ± 1.2*	78.9 ± 2.6
Diabetic (AdhVASH-1)	30.6 ± 0.6*	27.8 ± 0.9*	80.3 ± 2.0

*$P < 0.05$ vs. nondiabetic animals. Values are shown as means ±SE; $n = 5$ in each group. Vehicle, vehicle buffer treated; MBP, mean blood pressure.

RESULTS

Serum and hepatic levels of VASH-1 after adenoviral transfer. The serum levels of VASH-1 were at very low levels in the nondiabetic mice (immunoblot). The AdhVASH-1–injected diabetic mice exhibited significantly elevated serum VASH-1 levels compared with the AdLacZ injection at 4 weeks after the initial injections (Fig. 1*A*). Similarly, hepatic expression of VASH-1 was markedly elevated in the AdhVASH-1–injected diabetic mice (Fig. 1*A*). The mice receiving AdLacZ or AdhVASH-1 did not exhibit any deleterious side effects, and all the mice survived.

Nondiabetic male imprinting control region mice receiving AdhVASH-1 did not exhibit any inflammatory or pathological alterations in the lungs, livers, or kidneys (data not shown), and hypertension or proteinuria was not observed.

Changes in blood glucose, body weight, and kidney weight/body weight. Treatment with AdhVASH-1 did not exhibit therapeutic effects on hyperglycemia (Table 1). Body weight was significantly lower in all of the diabetic groups compared with nondiabetic animals, and AdhVASH-1 treatment did not significantly influence body weight. Diabetic animals exhibited a significantly greater kidney weight-to-body weight (KW-to-BW) ratio compared with nondiabetic mice (Fig. 1*B*). Injection of AdhVASH-1 resulted in a significantly decreased KW-to-BW ratio compared with the control diabetic mice (Fig. 1*B*).

Changes in blood pressure. There were no significant differences in blood pressure among nondiabetic animals, diabetic mice, and diabetic mice treated with adenoviral vectors (Table 1).

Changes in serum creatinine, Ccr, and urinary albumin excretion. Serum creatinine levels did not significantly differ among the experimental groups. Although control diabetic mice showed a marked elevation of the Ccr and UACR, AdhVASH-1 suppressed STZ-induced increase of Ccr and UACR (Fig. 1*C* and *D*).

Histology and morphometric analysis. Glomerular hypertrophy and mesangial matrix expansion were significantly inhibited by AdhVASH-1 compared with the control diabetic animals (Fig. 1*E–H*). Morphometric analysis (Fig.

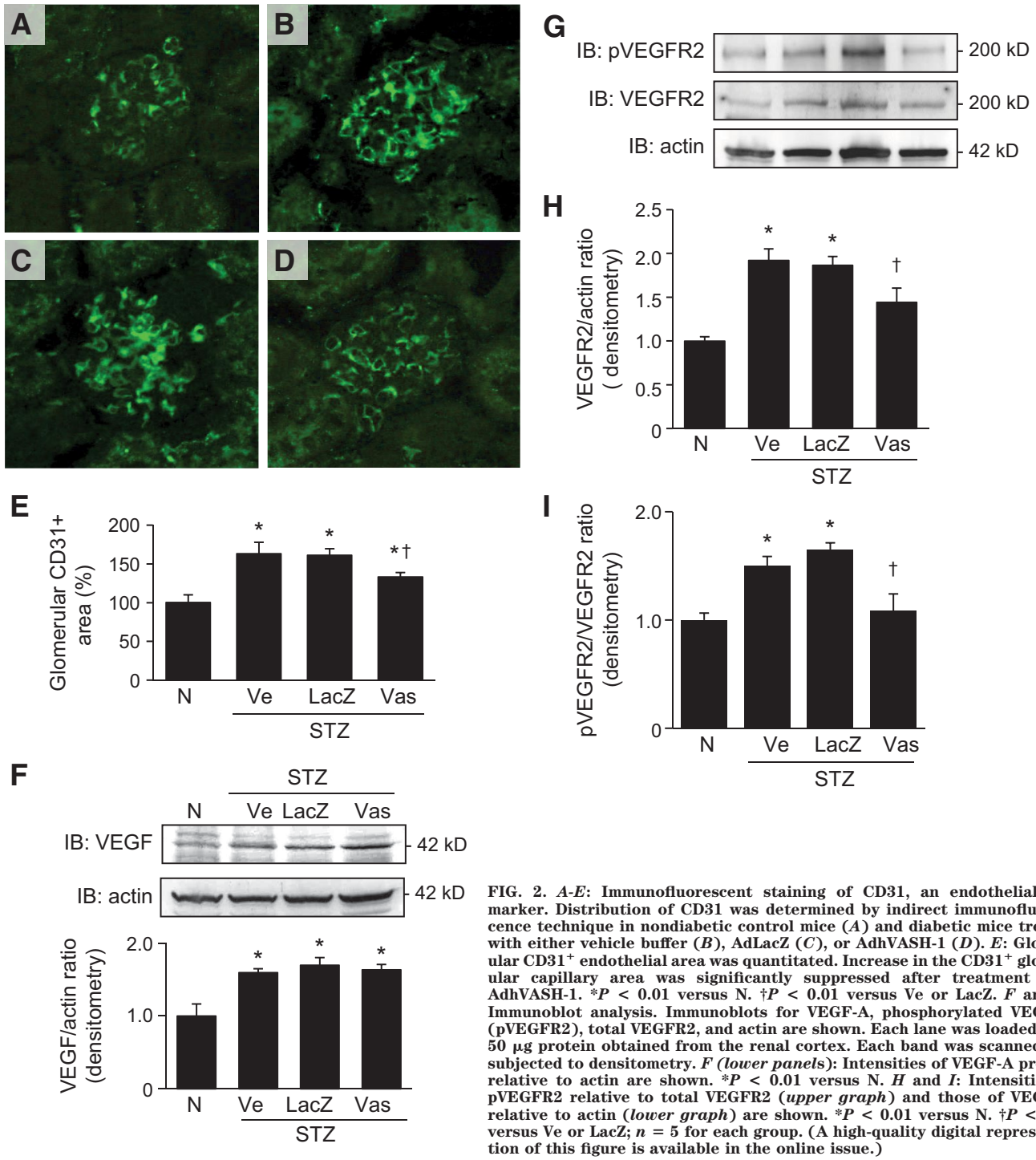

FIG. 2. ***A-E*****: Immunofluorescent staining of CD31, an endothelial cell marker. Distribution of CD31 was determined by indirect immunofluorescence technique in nondiabetic control mice (*A*) and diabetic mice treated with either vehicle buffer (*B*), AdLacZ (*C*), or AdhVASH-1 (*D*). *E*: Glomerular CD31$^+$ endothelial area was quantitated. Increase in the CD31$^+$ glomerular capillary area was significantly suppressed after treatment with AdhVASH-1. *P < 0.01 versus N. †P < 0.01 versus Ve or LacZ. *F* and *G*: Immunoblot analysis. Immunoblots for VEGF-A, phosphorylated VEGFR2 (pVEGFR2), total VEGFR2, 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 (lower panels)*: Intensities of VEGF-A protein relative to actin are shown. *P < 0.01 versus N. *H* and *I*: Intensities of pVEGFR2 relative to total VEGFR2 (*upper graph*) and those of VEGFR2 relative to actin (*lower graph*) are shown. *P < 0.01 versus N. †P < 0.05 versus Ve or LacZ; n = 5 for each group. (A high-quality digital representation of this figure is available in the online issue.)**

1*I* and *J*) further confirmed the inhibitory effects of AdhVASH-1 on these parameters.

Immunohistochemical analysis of CD31$^+$ endothelial area. We next evaluated differences in the amount of the CD31$^+$ glomerular endothelial area by immunofluorescence staining. In nondiabetic mice, CD31 was detected in glomerular capillaries (Fig. 2*A*), and increase of the CD31$^+$ area in glomeruli was observed in control diabetic mice (Fig. 2*B* and *C*). Treatment with AdhVASH-1 markedly suppressed the increase of the glomerular CD31$^+$ area (Fig. 2*D*). Quantitative analysis (Fig. 2*E*) further confirmed that the STZ-induced increase in the glomerular capillary area was significantly suppressed by AdhVASH-1. We evaluated the CD31$^+$ peritubular capillary (PTC) endothelial area to determine the potential adverse effects of VASH-1 on the survival of PTC. In the control diabetic mice, a slight increase of PTC density was observed, and AdhVASH-1 did not significantly influence the PTC density (supplemental Fig. 1, available in an online appendix).

Protein levels of VEGF-A and receptor VEGFR2 in renal cortex. The effect of AdhVASH-1 on the expression of proangiogenic factor VEGF-A and corresponding receptors VEGFR2 in the renal cortex was studied by immuno-

blot assay. The level of VEGF-A and VEGFR2 was significantly increased in the control diabetic mice, which is consistent with previous reports (9,15). Treatment with AdhVASH-1 did not affect the STZ-induced increase of VEGF-A but significantly suppressed the increase of VEGFR2 compared with AdLacZ (Fig. 2*F*–*H*).

Phosphorylation of VEGFR2 in vivo and in cultured hGECs. Next, we examined the potential inhibitory effects of VASH-1 on phosphorylation of VEGFR2. The ratio of phosphorylated VEGFR2 relative to total VEGFR2 in renal cortex of control diabetic mice was elevated compared with nondiabetic animals, and AdhVASH-1 treatment showed inhibitory effects (Fig. 2*G* and *I*). We performed cell culture analysis using hGECs. The ratio of phosphorylated VEGFR2 relative to total VEGFR2 in hGECs was significantly increased at 2–15 min after initiating stimulation with 1 nmol/l VEGF compared with control as detected by immunoblots (Fig. 3*A*). Because the peak of pVEGFR2-to-VEGFR2 ratio was observed at 5 min after stimulation with VEGF, we performed subsequent experiments under this condition. Increase in the levels of pVEGFR2-to-VEGFR2 ratio was significantly suppressed by recombinant VASH-1 as detected by immunoblots (Fig. 3*B*). Similarly, the level of pVEGFR2-to-VEGFR2 ratio in hGECs was significantly increased at 24 h after incubation in medium supplemented with 25 mmol/l glucose (high glucose) compared with incubation under the normal glucose condition (normal glucose) as detected by immunoblots (Fig. 3*C*). Addition of mannitol to the culture condition under normal glucose did not lead to the significant increase of pVEGFR2-to-VEGFR2 ratio, thus excluding the potential effect caused by elevated osmotic pressure. Treatment with recombinant VASH-1 resulted in the suppression of the increase of pVEGFR2-to-VEGFR2 ratio induced by high glucose in a dose-dependent manner (Fig. 3*C*).

Immunohistochemical analysis of glomerular type IV collagen. Next, the accumulation of glomerular type IV collagen was examined by immunofluorescence staining (Fig. 4). The amount of type IV collagen in glomeruli was increased in the control diabetic group (Fig. 4*B* and *C*) compared with the nondiabetic mice (Fig. 4*A*). 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 induced by STZ compared with AdLacZ treatment (Fig. 4*D*), and these results were further confirmed by quantitative morphometric analysis (Fig. 4*G*).

Immunohistochemical analysis of monocyte/macrophage infiltration. We examined glomerular infiltration of monocytes/macrophages by immunohistochemistry for F4/80. In the control diabetic mice, the number of F4/80(+) cells was significantly increased compared with the nondiabetic mice. Treatment with AdhVASH-1 markedly decreased the accumulation of monocytes/macrophages in glomeruli (Fig. 4*H*).

Protein and mRNA levels of TGF-β1 in renal cortex. TGF-β1 is a profibrotic factor involved in mesangial matrix expansion and renal hypertrophy in diabetic nephropathy (27). The control diabetic mice exhibited increased levels of TGF-β protein compared with the nondiabetic animals in the renal cortex (immunoblots). AdhVASH-1 significantly suppressed the increase of TGF-β in the diabetic animals compared with AdLacZ

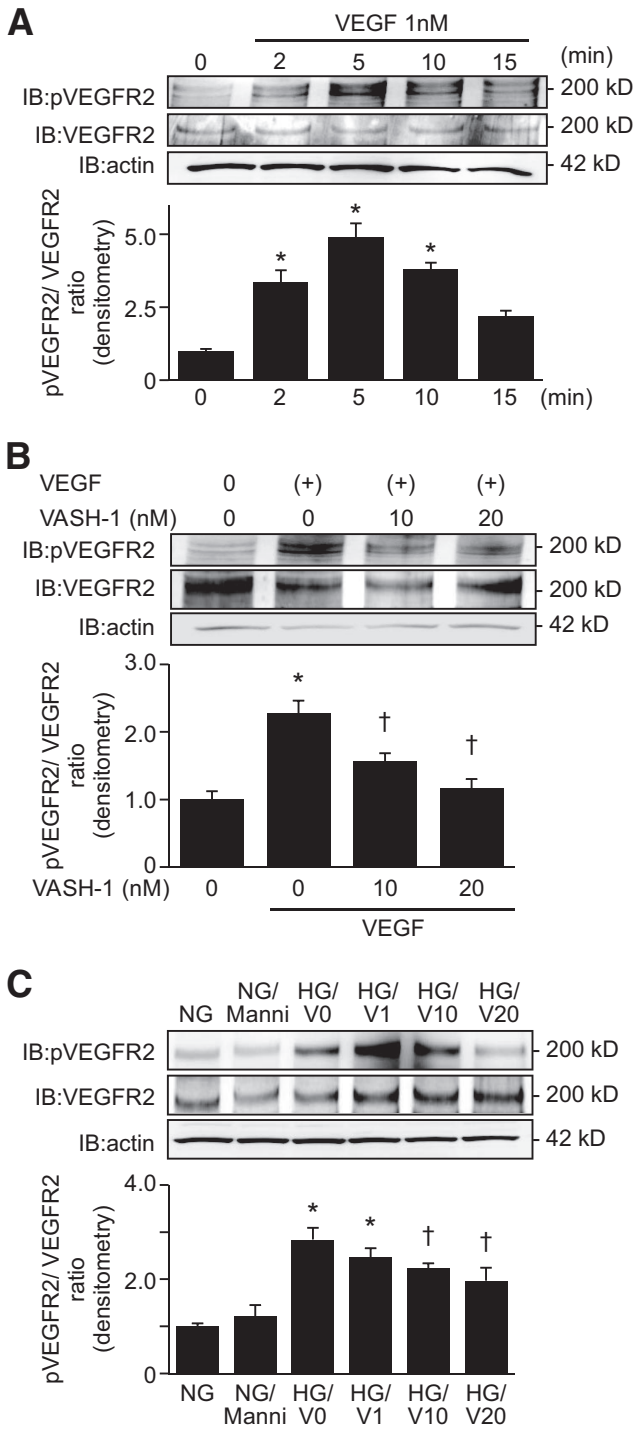

FIG. 3. *A*-*C*: Immunoblot analysis (cultured hGECs). Immunoblots for pVEGFR2, VEGFR2, and actin are shown. *A*: Cells were stimulated with 1 nmol/l of VEGF for 2–15 min. Intensities of pVEGFR2 protein relative to total VEGFR2 are shown. *$P < 0.01$ versus control (0). *B*: Cells were stimulated with 1 nmol/l of VEGF for 5 min in the presence of recombinant VASH-1 (0–20 nmol/l). Intensities of pVEGFR2 protein relative to VEGFR2 are shown. *$P < 0.01$ versus control. †$P < 0.05$ versus VEGF(+)/VASH-1(0). *C*: Cells were cultured under normal glucose (5.5 mmol/l) or high glucose (25 mmol/l) for 24 h in the presence of recombinant VASH-1 (0–20 nmol/l). Intensities of pVEGFR2 protein relative to VEGFR2 are shown. *$P < 0.01$ versus normal glucose or normal glucose/Manni. †$P < 0.05$ versus high glucose/V0. Each lane was loaded with 15 μg protein obtained from hGECs. Normal glucose+Manni, normal D-glucose plus D-mannitol (19.5 mmol/l); V0, without VASH-1; V1, 1 nmol/l VASH-1; V10, 10 nmol/l VASH-1; V20, 20 nmol/l VASH-1.

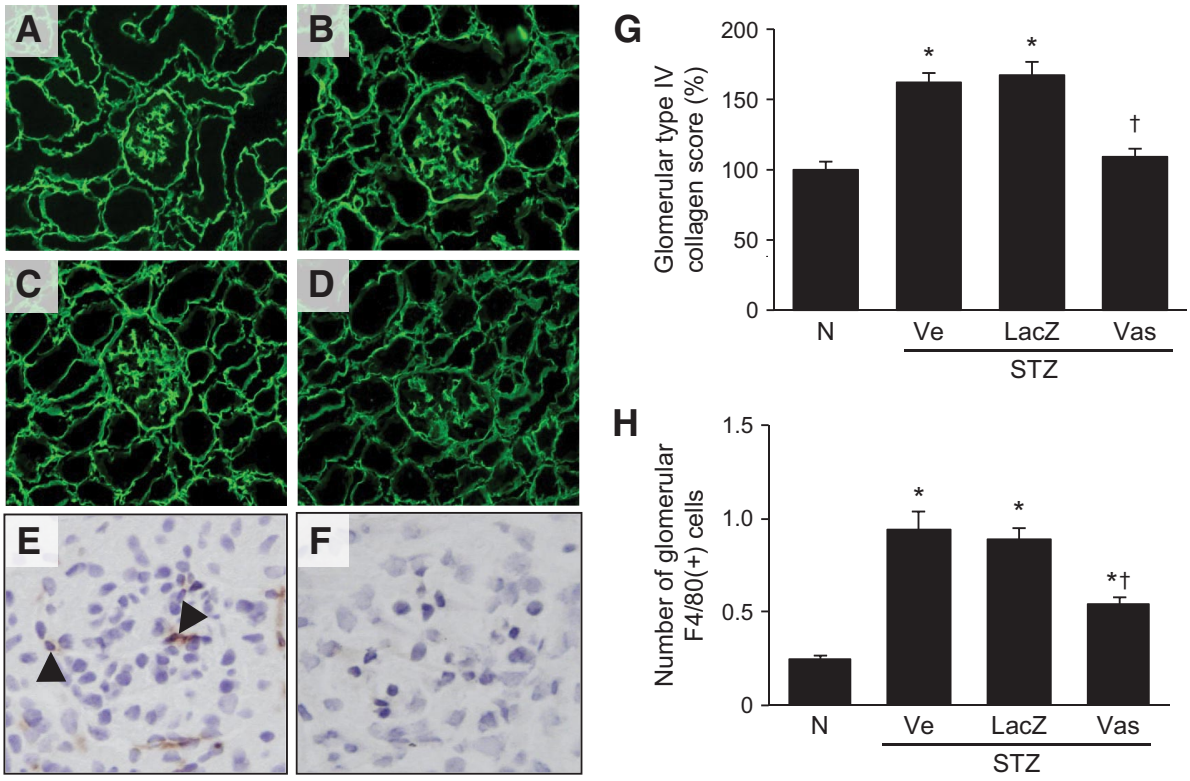

FIG. 4. *A–D*: Glomerular accumulation of type IV collagen was assessed by the indirect immunofluorescence method for nondiabetic control mice (*A*) and diabetic mice treated with either vehicle buffer (*B*), AdLacZ (*C*), or AdhVASH-1 (*D*). *A–D*: Original magnification ×200. *E* and *F*: Immunohistochemistry of F4/80$^+$ monocyte/macrophage. Representative light microscopic appearances of glomerulus in diabetic mice treated with vehicle buffer stained in the presence of the primary antibodies (*E*) or normal rat IgG (*F*) are shown. F4/80$^+$ cells were observed in diabetic mice (arrowheads; original magnification ×400). *G*: The amount of immunoreactive type IV collagen in glomeruli relative to the nondiabetic control group determined by computer image analysis is shown. *H*: The number of glomerular F4/80$^+$ monocyte/macrophages is shown. Increase in the F4/80$^+$ monocyte/macrophage number was significantly suppressed after treatment with AdhVASH-1; $n = 5$ for each group. *$P < 0.01$ versus N. †$P < 0.01$ versus Ve or LacZ. (A high-quality digital representation of this figure is available in the online issue.)

(Fig. 5*A* and *B*). Similarly, inhibitory effects of AdhVASH-1 on the levels of TGF-β1 mRNA were observed by real-time PCR (Fig. 5*C*).

Protein and mRNA levels of MCP-1 in renal cortex. MCP-1 is one of the crucial chemokines involved in the development of diabetic nephropathy (28). Control diabetic mice exhibited increased levels of MCP-1 protein compared with nondiabetic animals in the renal cortex (immunoblot). Treatment with AdhVASH-1 resulted in the suppression of MCP-1 protein levels compared with AdLacZ (Fig. 5*D* and *E*). Similar inhibitory effects of AdhVASH-1 on the increase of the mRNA levels of MCP-1 in diabetic animals were observed (Fig. 5*F*).

Protein levels of RAGE in renal cortex. The potential role of AGE in the pathogenesis of diabetic nephropathy has been reported (29). RAGE, a well-characterized cell surface receptor for AGE, plays an important role in the development of diabetic nephropathy (30). The control diabetic mice exhibited increased protein levels of RAGE compared with nondiabetic animals in the renal cortex (immunoblots). Treatment with AdhVASH-1 resulted in the suppression of RAGE protein levels compared with AdLacZ treatment (Fig. 5*G* and *H*).

Protein levels of TGF-β, MCP-1, and RAGE in cultured mouse mesangial cells. To examine the potential direct effects of VASH-1 on nonendothelial cells in association with the observed therapeutic effects in vivo, we performed cell culture analysis using primary mouse mesangial cells. The protein level of TGF-β, MCP-1, and RAGE in mesangial cells was significantly increased at 48 h after incubation in high glucose condition compared with incubation under normal glucose condition as detected by immunoblots (Fig. 6). Addition of mannitol to the normal glucose condition did not lead to the increase of TGF-β, MCP-1, and RAGE, thus excluding the potential effect by elevated osmotic pressure. Treatment with recombinant VASH-1 resulted in the suppression of the increase of protein levels for TGF-β, MCP-1, and RAGE induced by high glucose in a dose-dependent manner (Fig. 6).

Localization and the levels of endogenous mouse VASH-1 in kidney. In nondiabetic mice, endogenous mouse VASH-1 (mVASH-1) was observed in arterioles and in glomeruli. Immunoreactivity for mVASH-1 was increased in diabetic glomeruli, and was remarkable in the mesangial (α-SMA$^+$) area and in endothelial (CD31$^+$) area as detected by double immunofluorescent staining (Fig. 7*A* and *B*). In blood vessels, mVASH-1 was mainly localized to the outer aspect of vessels, presumably in smooth muscle cells (α-SMA$^+$) and in the adventitia, and slightly observed in the endothelium (CD31$^+$) as well (Fig. 7*A* and *B*). Immunoblot analysis revealed a mild increase of renal endogenous mVASH-1 in the vehicle-treated diabetic mice compared with the nondiabetic control mice without statistically significant difference (Fig. 2, available in an online appendix). Treatment with AdhVASH-1 did not alter the levels of endogenous mVASH-1 compared with the control diabetic mice.

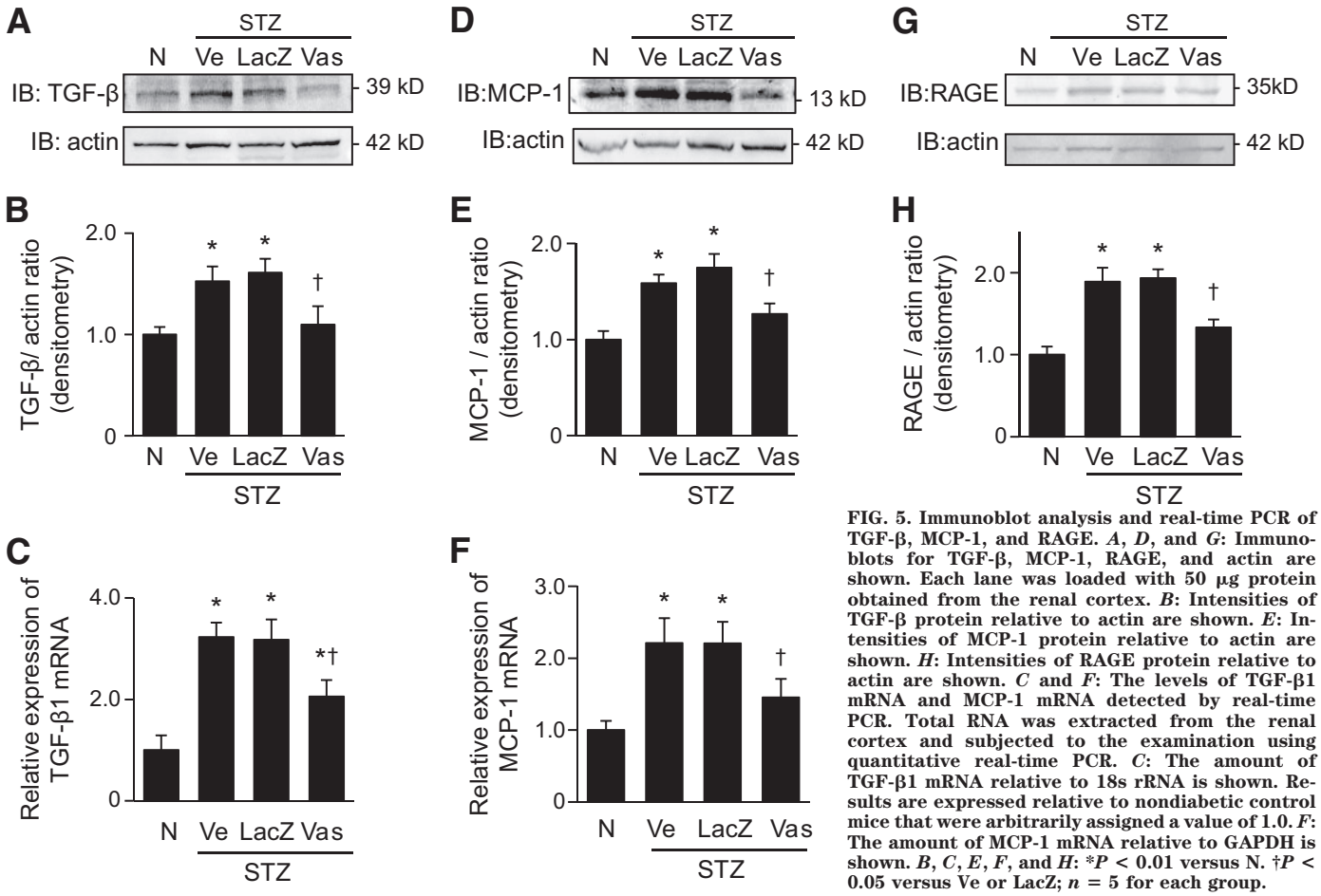

FIG. 5. Immunoblot analysis and real-time PCR of TGF-β, MCP-1, and RAGE. *A*, *D*, and *G*: Immunoblots for TGF-β, MCP-1, RAGE, and actin are shown. Each lane was loaded with 50 μg protein obtained from the renal cortex. *B*: Intensities of TGF-β protein relative to actin are shown. *E*: Intensities of MCP-1 protein relative to actin are shown. *H*: Intensities of RAGE protein relative to actin are shown. *C* and *F*: The levels of TGF-β1 mRNA and MCP-1 mRNA detected by real-time PCR. Total RNA was extracted from the renal cortex and subjected to the examination using quantitative real-time PCR. *C*: The amount of TGF-β1 mRNA relative to 18s rRNA is shown. Results are expressed relative to nondiabetic control mice that were arbitrarily assigned a value of 1.0. *F*: The amount of MCP-1 mRNA relative to GAPDH is shown. *B*, *C*, *E*, *F*, and *H*: *$P < 0.01$ versus N. †$P < 0.05$ versus Ve or LacZ; $n = 5$ for each group.

DISCUSSION

In the present study, we utilized a STZ-induced type 1 diabetes mouse model to demonstrate the therapeutic efficacy of VASH-1. Although renal failure is not easily reproducible, some of the characteristic early alterations in human diabetic nephropathy such as albuminuria, glomerular hyperfiltration, and some of the characteristic histopathologic changes can be observed in this model (31). Intravenous administration of AdhVASH-1 resulted in the sustained increase of serum levels of hVASH-1 presumably derived from liver, without causing any systemic inflammatory reactions. Additionally, administration of AdhVASH-1 in nondiabetic mice did not cause any inflammatory histological alterations in the kidney. Therefore, we consider the present approach using adenoviral vectors as nonharmful for experimental animals. Treatment with AdhVASH-1 did not affect hyperglycemia or body weight loss induced by STZ, similar to our previous observations using antiangiogenic tumstatin peptide and endostatin peptide (14,15). Although diabetic mice exhibited significant weight loss, the extent of reduced body weight was comparable to previous reports utilizing this model. In the control diabetic mice, characteristic alterations in early diabetic nephropathy such as albuminuria, glomerular hypertrophy, glomerular hyperfiltration as evidenced by increased Ccr, and renal hypertrophy were observed. These early abnormalities in diabetic nephropathy were significantly inhibited by AdhVASH-1 compared with AdLacZ treatment. Histological assessment demonstrated that treatment with AdhVASH-1 suppressed the increase of the $CD31^+$ glomerular endothelial area in diabetic mice. Experimental diabetic animals exhibit an increased glomerular filtration surface area as well as glomerular capillary number in the early stage of the disease (7,8). AdhVASH-1 treatment has suppressed these alterations potentially via the antiangiogenic efficacy, leading to the observed therapeutic effects on the increase of Ccr and albuminuria.

In the present study, the level of VEGF-A was increased in the renal cortex of diabetic mice, consistent with previous studies (9,10,14). Recent reports have demonstrated reduced expression of VEGF-A in human diabetic nephropathy patients (32,33). Although diabetic animal models are often studied in a relatively early phase of the disease, most diabetic patients used in clinical studies were already in a moderately advanced stage. For instance, reduced VEGF expression in the renal interstitium was associated with interstitial vascular rarefaction in a study using samples of human diabetic nephropathy (33), but we could not detect a reduction of PTC density in the present study in the control diabetic animals. Treatment with AdhVASH-1 did not diminish the increase of VEGF-A in the renal cortex but resulted in the suppression of the increase of VEGFR2. The regulatory role of VASH-1 on VEGFR2 but not VEGF-A is consistent with previous results on proliferative retinopathy (21). In addition, VASH-1 significantly suppressed the increase of pVEGFR2-to-VEGFR2 ratio in diabetic animals as well as in cultured glomerular endothelial cells. Our results are not consistent with previous reports demonstrating that VASH-1 did not inhibit VEGF-induced VEGFR2 phosphorylation in human umbilical vein endothelial cells (19). Unique characteris-

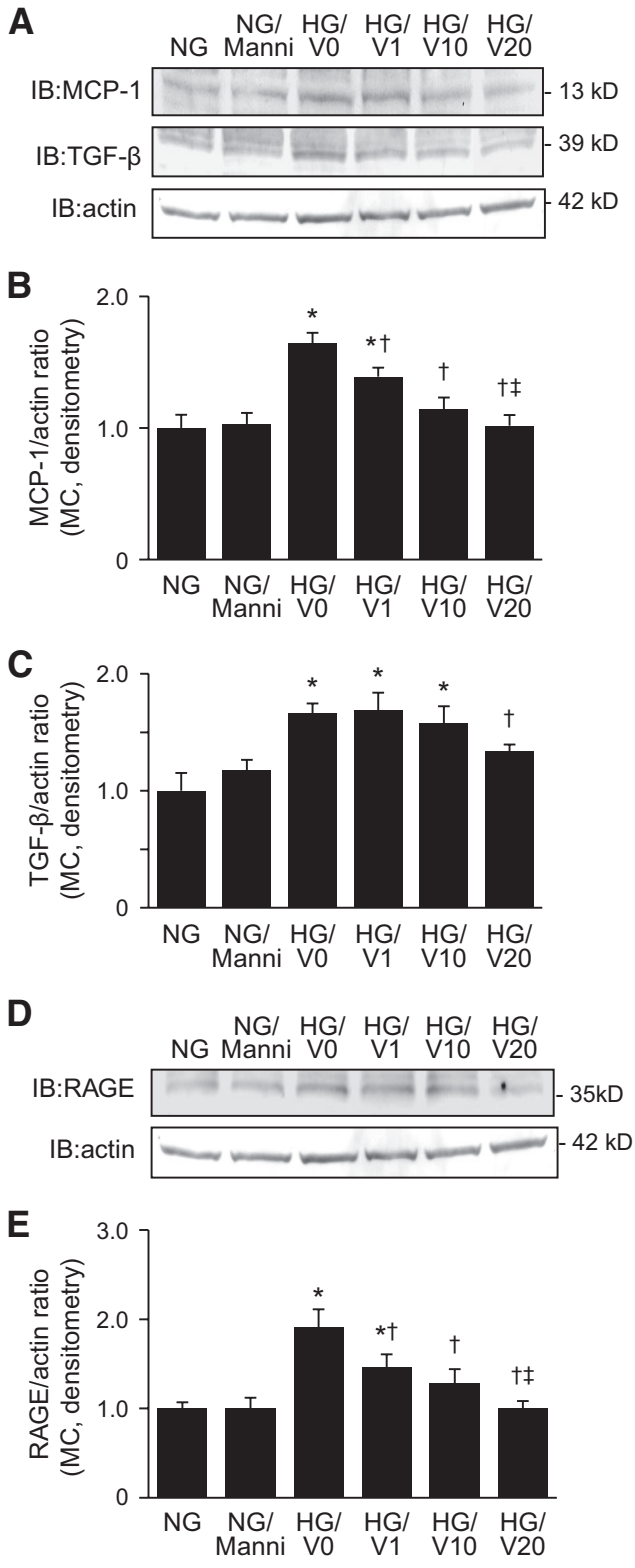

FIG. 6. *A-E*: Immunoblot analysis (cultured mesangial cells). *A* and *D*: Immunoblots for MCP-1, TGF-β, RAGE, and actin are shown. Each lane was loaded with 20 μg protein obtained from mouse mesangial cells. *B*: Intensities of MCP-1 protein relative to actin are shown. *$P < 0.05$ versus normal glucose (NG) or normal glucose/Manni. †$P < 0.05$ versus high glucose (HG)/V0. ‡$P < 0.05$ versus high glucose/V1. *C*: Intensities of TGF-β protein relative to actin are shown. *$P < 0.05$ versus normal glucose or normal glucose/Manni. †$P < 0.05$ versus high glucose/V0 or high glucose/V1. *E*: Intensities of RAGE protein relative to actin are shown. *$P < 0.05$ versus normal glucose or normal glucose/Manni. †$P < 0.05$ versus high glucose/V0. ‡$P < 0.05$ versus high glucose/V1.

tics of the glomerular endothelial cells possessing fenestration are well known, and such differences among distinct endothelial cell types may underlie the discrepant response to VASH-1 concerning VEGFR-2 phosphorylation. The therapeutic effect of VASH-1 in early diabetic nephropathy, at least in part, may be attributed to the inhibition of overactivation of the VEGF-A pathway in analogy with previous studies utilizing neutralizing anti-VEGF-A antibodies (11,12) and a recent report demonstrating that inducible overexpression of soluble flt-1 (VEGFR1), an antagonist of VEGF-A, in podocytes ameliorated diabetic glomerular alterations in mice (34). To date, cell surface receptors for VASH-1 as well as potential influence of VASH-1 on intracellular signal transduction have not been fully identified, and future analysis on crosstalk between VASH-1 signaling and VEGF signaling is required.

We observed a significant inhibitory effect of AdhVASH-1 on renal hypertrophy in diabetic mice. VEGF-A augments protein synthesis and hypertrophy in renal proximal tubular epithelial cells (35). Considering the dominant contribution of the tubular compartment in organizing renal mass, we speculate that AdhVASH-1 might have affected the tubular hypertrophy potentially via regulating VEGF-A–mediated signaling.

In the present study, increase in the levels of renal TGF-β was significantly suppressed by AdhVASH-1, potentially associated with therapeutic efficacy on the accumulation of ECM. These results are consistent with previous reports demonstrating the inhibitory effects of antiangiogenic reagents on ECM accumulation in animal models of diabetic nephropathy (14–16,18,36).

Interestingly, recombinant VASH-1 treatment resulted in the suppression of the increase of TGF-β induced by high glucose in cultured mesangial cells. We previously observed similar inhibitory effects of NM-3 on high glucose–induced TGF-β production in mesangial cells (18), suggesting the novel potential therapeutic mechanisms of antiangiogenic factors mediated via direct interaction with 'nonendothelial' mesangial cells.

A recent report has demonstrated the potential role of VEGF in mediating glomerular monocyte/macrophage infiltration in a diabetic animal model (37). Therefore, observed anti-inflammatory effect of VASH-1 might be, at least in part, associated with regulation of vascular permeability. Yamashita et al. (20) have demonstrated the therapeutic role of AdhVASH-1 in preventing arterial neointimal formation. Adventitial macrophage infiltration was inhibited by AdhVASH-1 (20), similar to our present results. In addition, VASH-1 suppressed renal MCP-1 expression in diabetic animals and suppressed the increase of MCP-1 induced by high glucose in cultured mesangial cells. The inhibitory effect of AdhVASH-1 on the increase of chemokines may also mediate its anti-inflammatory effects. Previous reports have demonstrated the association between infiltration of macrophages and the accumulation of ECM proteins in the mesangium in diabetic models (38,39). Regarding the source of TGF-β, macrophages as well as mesangial cells can secrete TGF-β (40). Thus, we speculate that the observed regulatory effects of VASH-1 on the accumulation of monocytes/macrophages might have contributed to the inhibition of mesangial matrix accumulation.

The deleterious effects of AGE in the development of diabetic nephropathy and the therapeutic effects of AGE inhibitors in preventing the progression of experimental

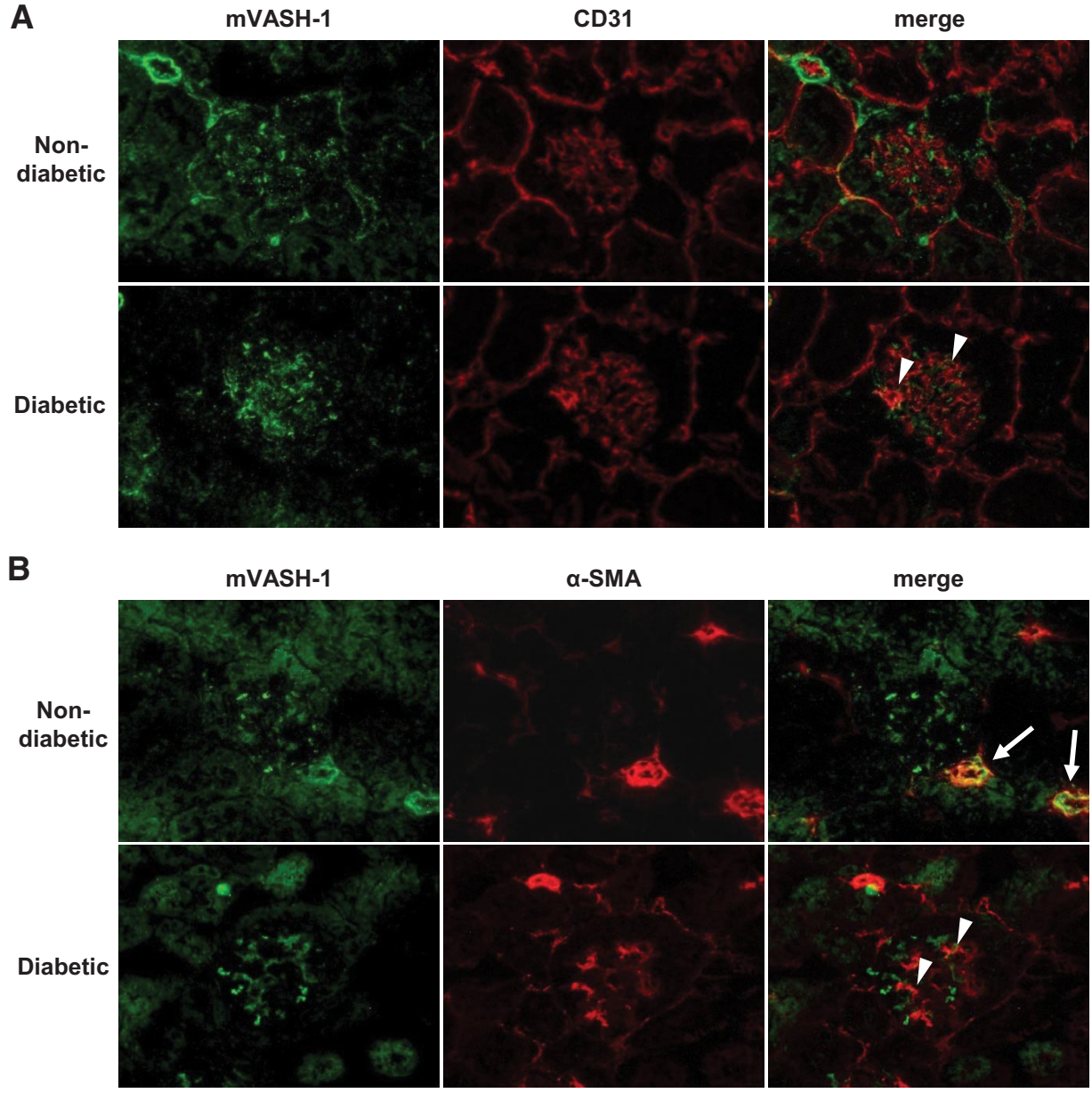

FIG. 7. Double immunofluorescent staining for endogenous mVASH-1, CD31, and α-SMA. *A*: Double immunofluorescent staining of mVASH-1 (green), CD31 (red), and merged images in the kidney from nondiabetic (*upper panels*) or diabetic (*lower panels*) mice. Although mVASH-1 was faintly observed in nondiabetic glomeruli, increased immunoreactivity for mVASH-1 was observed and partially colocalized with the CD31$^+$ endothelial cells (arrowheads) in the control diabetic mice. *B*: Double immunofluorescent staining of mVASH-1 (green), α-SMA (red), and merged images in the kidney from nondiabetic mice (*upper panels*) or control diabetic animals (*lower panels*). In the nondiabetic kidney, immunoreactivity for α-SMA was observed in extraglomerular arterioles colocalized with mVASH-1 (arrows). Immunoreactivity of mVASH-1 partially colocalized with the α-SMA$^+$ mesangial cells (arrowheads) in the control diabetic mice. Original magnification ×400. (A high-quality digital representation of this figure is available in the online issue.)

diabetic nephropathy were previously reported (10,41). AGE induces overexpression of TGF-β, MCP-1, and VEGF in cultured mesangial cells (42,43). Interaction of AGE with RAGE plays an important role in diabetic nephropathy (44). VASH-1 significantly suppressed renal RAGE levels in diabetic mice and suppressed the increase of RAGE induced by high glucose in cultured mesangial cells. These results suggest that antifibrotic and anti-inflammatory effects of VASH-1 were partially mediated via downregulating RAGE levels in mesangial cells, resulting in the suppression of TGF-β and MCP-1.

Regarding endogenous levels and localization of mVASH-1, renal protein levels of mVASH-1 were mildly increased in diabetic control mice. AdhVASH-1 did not alter renal mVASH-1 levels compared with AdLacZ treatment. In diabetic mice, mVASH-1 was observed in the glomerular endothelium and mesangial area. Previous reports have revealed the localization of human VASH-1 in endothelium of the placenta, endometria, brain, atherosclerotic lesions, and choroidal neovascular membranes (19,20,45,46). mVASH-1 was expressed in the retinal endothelial cells in mice with ischemic retinopathy (21). The localization of mVASH-1 in the mesangial area suggests the synthesis of VASH-1 in mesangial cells, but also the potential mesangial deposition of VASH-1 released from endothelial cells could not be excluded. Studies using VASH-1–deficient mice (47) may clarify biological roles of endogenous mVASH-1 in diabetic nephropathy in future.

There are several limitations to the present study. Systemic administration of antiangiogenic reagents may lead to reduced angiogenic response in a setting requiring neovessel formation such as myocardial infarction and limb ischemia, pathological conditions complicated in advanced diabetic patients. Neointimal formation is the crucial cause of luminal narrowing of arteries in atherosclerosis. Although further assessment in the advanced stage is required, AdhVASH-1 might be tolerable or even therapeutic for atherosclerotic conditions considering a previous report showing the therapeutic effects of AdhVASH-1 in preventing neointimal formation (20). The crucial involvement of chronic hypoxia in association with reduction of PTC in progressing tubulointertstitial injuries has been reported (48). 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 in advanced diabetic nephropathy might be required.

Considering application to diabetic patients, potential adverse events accompanied by the injection of adenoviral vectors such as nonspecific inflammatory reactions and the replication of adenoviruses in vivo should be avoided, and further assessments on the safety of this strategy are required.

Recently, Eremina et al. (49) have demonstrated the potential adverse events of bevacizumab, a humanized monoclonal antibody against VEGF, resulting in thrombotic microangiopathy in patients with cancer. Considering the lack of such histological alterations in previous experimental studies using anti-VEGF antibodies or SU5416 (11–13), anti-VEGF therapy might not be detrimental for patients with diabetic nephropathy. More recently, Ku et al. (34) have demonstrated that inducible podocyte-specific overexpression of sVEGFR1 in adult mice ameliorated diabetic glomerular alterations, suggesting the involvement of VEGF-A in diabetic nephropathy. Although VASH-1 suppresses overactivation of VEGFR2, it does not serve as a specific inhibitor of VEGF signaling (19). In addition, AdhVASH-1 did not cause proteinuria in nondiabetic mice. Thus, VASH-1 treatment is distinct from strategies with specific inhibition of VEGF potentially associated with less adverse events.

In conclusion, we demonstrated that VASH-1 effectively ameliorated alterations in an animal model of early diabetic nephropathy. Our results demonstrate the direct effects of VASH-1 on glomerular endothelial and mesangial cells in association with antiangiogenic, antifibrotic, and anti-inflammatory mechanisms and regulatory effects on RAGE-mediated pathways. 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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