ADAMTS13 Retards Progression of Diabetic Nephropathy by Inhibiting Intrarenal Thrombosis in Mice

Nirav Dhanesha, Prakash Doddapattar, Mehul R. Chorawala, Manasa K. Nayak, Koichi Kokame, Janice M. Staber, Steven R. Lentz, Anil K. Chauhan

Objective—ADAMTS13 (a disintegrin and metalloprotease with thrombospondin type I repeats-13) prevents microvascular thrombosis by cleaving prothrombogenic ultralarge von Willebrand factor (VWF) multimers. Clinical studies have found association between reduced ADAMTS13-specific activity, ultralarge VWF multimers, and thrombotic angiopathy in patients with diabetic nephropathy. It remains unknown, however, whether ADAMTS13 deficiency or ultralarge VWF multimers have a causative effect in diabetic nephropathy.

Approach and Results—The extent of renal injury was evaluated in wild-type (WT), Adamts13−/− and Adamts13−/−Vwf−/− mice after 26 weeks of streptozotocin-induced diabetic nephropathy. We found that WT diabetic mice exhibited low plasma ADAMTS13-specific activity and increased VWF levels (P<0.05 versus WT nondiabetic mice). Adamts13−/− diabetic mice exhibited deterioration of kidney function (increased albuminuria, plasma creatinine, and urea; P<0.05 versus WT diabetic mice), independent of hyperglycemia and hypertension. Deterioration of kidney function in Adamts13−/− diabetic mice was concomitant with aggravated intrarenal thrombosis (assessed by plasminogen activator inhibitor, VWF, fibrinogen, and CD41-positive microthrombi), increased mesangial cell expansion, and extracellular matrix deposition (P<0.05 versus WT diabetic mice). Genetic deletion of VWF in Adamts13−/− diabetic mice improved kidney function, inhibited intrarenal thrombosis, and alleviated histological changes in glomeruli, suggesting that exacerbation of diabetic nephropathy in the setting of ADAMTS13 deficiency is VWF dependent.

Conclusions—ADAMTS13 retards progression of diabetic nephropathy, most likely by inhibiting VWF-dependent intrarenal thrombosis. Alteration in ADAMTS13–VWF balance may be one of the key pathophysiological mechanisms of thrombocytopenia in diabetes mellitus.

Visual Overview—An online visual overview is available for this article. (Arterioscler Thromb Vasc Biol. 2017;37:1332-1338. DOI: 10.1161/ATVBAHA.117.309539.)

Key Words: albuminuria ■ diabetic nephropathy ■ streptozotocin ■ thrombosis ■ von Willebrand factor

Diabetic nephropathy is the major cause of end-stage renal disease, contributing to ≥40% of new cases, and it is an independent risk factor for cardiovascular diseases. Current strategies to treat diabetic nephropathy include glicemic control with antidiabetic drugs and antihypertensive medications, such as angiotensin-converting enzyme inhibitors. Despite therapy, several patients remain at high risk because of adverse events.

Chronic hyperglycemia and oxidative stress—induced endothelial dysfunction and platelet activation are key features of progressive diabetic nephropathy. Oxidative stress was shown to be associated with accumulation of ultralarge von Willebrand factor (ULVWF) multimers and thrombotic angiopathy in patients with diabetic nephropathy. Notably, ULVWF multimers, which are stored in platelet α-granules and endothelial Weibel–Palade bodies, are extremely large (up to 20,000 kDa) and are considered to be prothrombogenic.

ULVWF multimers are not present in the plasma of healthy individuals because, upon release from platelets or endothelial cells, they are rapidly cleaved by the plasma protease ADAMTS13 (a disintegrin and metalloprotease with thrombospondin type I repeats-13) into less active, smaller VWF multimers. ADAMTS13 is synthesized primarily by hepatic stellate cells and to a lesser extent by endothelial cells, megakaryocytes, and podocytes. Deficiency of ADAMTS13 or low levels of ADAMTS13 (≤10%) increases plasma levels of ULVWF and causes thrombotic thrombocytopenic purpura, a disorder of thrombotic microangiopathy. Recent clinical studies suggest that chronic hyperglycemia can imbalance ADAMTS13–VWF axis, and, thereby may potentially contribute to thrombotic angiopathy in the setting of diabetes mellitus. Despite these epidemiological findings, which predict that ADAMTS13–VWF axis imbalance may be causally linked to adverse renal and cardiovascular events.
in patients with diabetes mellitus, experimental evidence for causality is lacking.

Herein, we determined the role of the ADAMTS13–VWF axis in the progression of diabetic nephropathy by testing the hypothesis that ADAMTS13 deficiency exacerbates diabetic nephropathy by aggravating intrarenal thrombosis mediated by ULVWF. We used Adamts13−/− (model of ULVWF multimers) and Adamts13−/−Vwf−/− mice in an experimental model of streptozotocin-induced diabetic nephropathy. We found that ADAMTS13 deficiency exacerbated diabetic nephropathy, whereas deletion of VWF alleviated the aggravated diabetic nephropathy in Adamts13−/− mice.

## Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

## Results

### Wild-Type Diabetic Mice Exhibit Reduced Plasma ADAMTS13-Specific Activity and Increased VWF Levels

Reduced ADAMTS13-specific activity and elevated VWF levels have been reported in the plasma of patients with diabetic nephropathy. We determined whether the progression of diabetic nephropathy in streptozotocin-induced diabetes mellitus model would cause an increase in VWF levels associated with decreased ADAMTS13-specific activity. In wild-type (WT) diabetic mice, we found that plasma ADAMTS13-specific activity was markedly reduced by ~46%, whereas plasma VWF levels were increased by 3.8-fold (P<0.05 versus WT nondiabetic mice, Figure 1A and 1B).

### ADAMTS13 Deficiency Worsens Kidney Function During Progression of Diabetic Nephropathy

Next, we determined whether ADAMTS13 deficiency exacerbates diabetic nephropathy. As expected, induction of diabetes mellitus in WT mice significantly deteriorated kidney function as assessed by increased urinary albumin secretion, plasma creatinine, and urea levels concomitant with an increase in kidney weight (P<0.05 versus WT nondiabetic mice, Figure 2). Adamts13−/− diabetic mice exhibited further deterioration in kidney function compared with WT diabetic mice (P<0.05, Figure 2), which was independent of hyperglycemia and hypertension (Figure SI through SIII in the online-only Data Supplement).

### ADAMTS13 Deficiency Aggravates Intrarenal Thrombosis and Worsens Key Histological Features of Diabetic Nephropathy

ADAMTS13 is known to prevent thrombus formation in the microvasculature by cleaving prothrombotic ULVWF multimers. We hypothesized that ADAMTS13 deficiency aggravates intrarenal thrombosis, and thereby exacerbates diabetic nephropathy. To evaluate intrarenal thrombosis, we quantified intrarenal fibrin(ogen) deposition, VWF, plasminogen activator inhibitor (PAI-1), and CD41 levels in kidney homogenates. We found that intrarenal fibrin(ogen) and CD41 levels (determined by Western blot) were significantly increased in Adamts13−/− diabetic mice compared with WT diabetic mice (P<0.05; Figure 3A through 3C). Consistent with these findings, PAI-1 mRNA expression levels were increased in kidney homogenates of Adamts13−/− diabetic mice (P<0.05 versus WT diabetic mice; Figure SIV in the online-only Data Supplement). Furthermore, we found an increase in intravascular platelet microthrombi (CD41-positive platelet deposits) in the glomeruli of Adamts13−/− diabetic mice (P<0.05 versus WT diabetic mice, Figure 3D). Together, these results suggest that ADAMTS13 deficiency exacerbates intrarenal thrombosis. Next, we evaluated mesangial cell expansion and extracellular matrix (ECM) deposition (fibronectin and collagen) in the kidney glomeruli, 2 key histological features of diabetic nephropathy. Adamts13−/− diabetic mice exhibited significantly increased glomerular mesangial cell expansion as assessed by periodic acid Schiff staining (P<0.05 versus WT diabetic mice, Figure 4A). Concomitantly, Adamts13−/− diabetic mice showed increased ECM deposition and fibrosis as evident by increased fibronectin and collagen accumulation (P<0.05 versus WT diabetic mice, Figure 4B and 4C). Nondiabetic WT or Adamts13−/− mice showed minimal changes in mesangial cell expansion and ECM deposition (Figure 4). Next, we determined inflammatory cytokines interleukin-1β and tumor necrosis factor-α levels in kidney extracts. Although interleukin-1β and tumor necrosis factor-α levels were significantly increased in both WT and Adamts13−/− diabetic mice when compared with respective nondiabetic mice, they were comparable between WT diabetic and Adamts13−/− diabetic mice (Figure SV in the online-only Data Supplement).

### Genetic Deletion of VWF in Adamts13−/− Mice Improves Kidney Function by Inhibiting Intrarenal Thrombosis and Alleviating Key Hallmark Features of Diabetic Nephropathy

To determine the molecular mechanism by which ADAMTS13 deficiency contributes to diabetic nephropathy exacerbation, we focused on VWF because it is the only known substrate for ADAMTS13 in multiple experimental models. We hypothesized that genetic deletion of VWF in Adamts13−/− mice will alleviate diabetic nephropathy exacerbation. Blood glucose (Figure SIA in the online-only Data Supplement), body weight (Figure SIB in the online-only Data Supplement), diabetes mellitus incidence rate (Figure SII in the online-only Data Supplement).
Supplement), systolic blood pressure (Figure SIII in the online-only Data Supplement), and inflammatory cytokines (Figure SV in the online-only Data Supplement) were comparable between diabetic WT, \textit{Adamts}13−/−, and \textit{Adamts}13−/−\textit{Vwf}−/−mice. Despite this, we found that genetic ablation of VWF in \textit{Adamts}13−/−diabetic mice markedly improved kidney function, inhibited intrarenal thrombosis, and alleviated histological changes in glomeruli ($P<0.05$ versus \textit{Adamts}13−/−diabetic mice, Figures 2 through 4). Together, these results strongly suggest that exacerbation of diabetic nephropathy observed in the setting of ADAMTS13 deficiency is VWF dependent.

Discussion
The key novel findings of this study are (1) diabetes mellitus in mice results in increased VWF levels that are associated with decreased ADAMTS13-specific activity, (2) severe ADAMTS13 deficiency in mice exacerbates diabetic nephropathy, likely mediated, in part, due to increased intrarenal thrombosis, and (3) exacerbation of diabetic nephropathy observed in the setting of ADAMTS13 deficiency is VWF dependent.

We found that WT diabetic mice have significantly low levels of ADAMTS13-specific activity and elevated levels of VWF in the plasma compared with nondiabetic littermates. Our findings are consistent with several population-based

Figure 1. Wild-type (WT) diabetic mice exhibited reduced ADAMTS13 (a disintegrin and metalloprotease with thrombospondin type I repeats-13)–specific activity and increased von Willebrand factor (VWF) levels. Plasma VWF and ADAMTS13 levels were determined after 26 weeks of streptozotocin-induced diabetic nephropathy. A. Plasma ADAMTS13–specific activity. B. Plasma VWF levels. Data are presented as means±SEM. $n=5$ to 8 mice/group. Statistical analysis: unpaired $t$ test.

Figure 2. Deterioration of renal function in the \textit{Adamts}13−/−diabetic mice was von Willebrand factor (VWF) dependent. A. Urine albumin/creatinine ratio. B. Kidney weight/body weight. C. Plasma creatinine. D. Plasma urea. Data are presented as means±SEM. $n=5$ to 12 mice/group. Albuminuria, kidney weight, plasma creatinine, and urea were significantly increased in diabetic mice when compared with nondiabetic mice in all the groups ($P<0.05$). Statistical analysis: 1-way ANOVA followed by Holm–Sidak multiple comparison test. NS indicates nonsignificant; and WT, wild-type.
studies, which have observed low ADAMTS13 activity and high VWF antigen levels in type 1 and type 2 diabetic patients affected with diabetic nephropathy. The mechanisms by which ADAMTS13-specific activity is reduced in the setting of diabetes mellitus remain unclear. Possible mechanisms include: (1) reduced synthesis and secretion of ADAMTS13 by hepatic stellate cells in the liver; (2) increased ADAMTS13 consumption or incorporation into thrombi together with its substrate VWF, as observed in patients with acute myocardial infarction; (3) loss of function due to inhibitory autoantibodies to ADAMTS13; and (4) oxidative modification of ADAMTS13.

Clinical studies have suggested that oxidative stress in type 2 diabetic patients affected with diabetic nephropathy is associated with increased ULVWF multimers. However, to date there is no experimental evidence to suggest a causal role for ULVWF multimers in the exacerbation of diabetic nephropathy. One can speculate that ULVWF multimers...
might be simply an associated marker of disease status, possibly secondary to endothelial cell dysfunction, and oxidative stress. We used \textit{Adamts13}−/− and \textit{Adamts13}−/−\textit{Vwf}−/− mice to determine the role of the ADAMTS13–VWF axis in experimental diabetic nephropathy. Herein, we provide evidence that \textit{Adamts13}−/− diabetic mice are more susceptible to diabetic nephropathy and have deteriorated kidney function. Because VWF is the only known substrate for ADAMTS13,13,16,17 we determined whether exacerbated diabetic nephropathy in \textit{Adamts13}−/− mice is VWF dependent. We found that genetic deletion of VWF in \textit{Adamts13}−/− mice alleviated exacerbated diabetic nephropathy. Together, these results strongly suggest a causal role for the ADAMTS13–VWF axis in the progression of diabetic nephropathy.

It is intriguing to consider possible mechanisms by which the ADAMTS13–VWF axis may contribute to the progression of diabetic nephropathy. Because podocytes and glomerular endothelial cells synthesize ADAMTS13, we hypothesized that ADAMTS13 deficiency may aggravate intrarenal thrombosis in the high shear environment of glomeruli due to the accumulation of platelet-rich microthrombi, and, thereby exacerbate diabetic nephropathy. Spontaneous thrombus formation has been demonstrated in activated microvessels of \textit{Adamst13}−/− mice, but not in WT mice.15 Consistent with this hypothesis, we found that thrombosis markers such as PAI-1, VWF, fibrinogen, and CD41 levels were significantly elevated in kidney homogenates of \textit{Adamts13}−/− diabetic mice, suggesting aggravated intrarenal thrombosis. Consistent with these results, we found an increase in intravascular platelet microthrombi (CD41-positive platelet deposits) in the glomeruli of \textit{Adamts13}−/− diabetic mice. On basis of these results, we suggest that ADAMTS13, by regulating ULVWF/VWF multimer size, may inhibit thrombotic angiopathy in the setting of diabetes mellitus. Indeed, a recent study has shown that

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### Figure 4

Increased mesangial cell expansion and extracellular matrix deposition in the glomeruli in \textit{Adamts13}−/− diabetic mice were von Willebrand factor (VWF) dependent. **Left**, Representative images of (A) periodic acid Schiff (PAS)–stained (black arrow) kidney section, (B) fibronectin-stained (red arrow) kidney section, and (C) picrosirius red (yellow arrow)–stained kidney section. **Right**, Semiquantitative score for the respective staining. \(P<0.05\) for diabetic mice when compared with nondiabetic mice in all the groups. Data are presented as means±SEM. Mean score of 5 to 20 glomeruli from each mouse were taken from 5 to 6 mice/group. Statistical analysis: 1-way ANOVA followed by Holm–Sidak multiple comparison test. NS indicates nonsignificant; and WT, wild-type.

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**Table 1**

<table>
<thead>
<tr>
<th>Condition</th>
<th>\textit{Adamts13}−/−</th>
<th>\textit{Adamts13}−/−\textit{Vwf}−/−</th>
<th>Non diabetic</th>
<th>Diabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAS-stained</td>
<td></td>
<td></td>
<td>(A)</td>
<td>(B)</td>
</tr>
<tr>
<td>Fibronectin-stained</td>
<td></td>
<td></td>
<td>(C)</td>
<td></td>
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<tr>
<td>Picrossirius red-stained</td>
<td></td>
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recombinant ADAMTS13 inhibits thrombotic microangiopathy after systemic vascular endothelial growth factor inhibition.24 We and others have demonstrated that in addition to its role in thrombosis, the ADAMTS13–VWF axis also functions in the modulation of inflammatory responses in experimental models of stroke, myocardial infarction, and atherosclerosis.10,18,25,26 However, to our surprise, we found that the inflammatory cytokines interleukin-1β and tumor necrosis factor-α were comparable in kidney extracts of Adamts13−/− diabetic and WT diabetic mice, suggesting that most likely it is the aggravation of intrarenal thrombosis that mediates diabetic nephropathy exacerbation in Adamts13−/− mice in the setting of diabetes mellitus.

It is known that PAI-1 contributes to diabetic nephropathy exacerbation by regulating TGF-β (transforming growth factor-beta) and renal production of ECM proteins, such as collagen and fibronectin.27 Accumulation of ECM proteins and mesangial cell expansion in the kidney glomerular and tubular compartments are hallmarks of diabetic nephropathy and worsening of renal function. We found that ADAMTS13 deficiency in mice promotes mesangial cell expansion and accumulation of collagen and fibronectin in the glomerular compartment, which was associated with increased PAI-1 protein levels. Although endothelial cells are the major source of PAI-1, platelets also contain significant amounts of PAI-1. We speculate that the source of increased PAI-1 levels in kidney homogenates of Adamts13−/− mice is both endothelial cells and activated platelets within thrombi. On the basis of our findings, we proposed a mechanistic model in which ADAMTS13 deficiency combined with hyperglycemia aggravates intrarenal thrombosis. Increased PAI-1 levels are released from activated endothelial cells and platelet-rich thrombi in the tubular microenvironment, which, in turn, potentiate TGF-β production to enhance the production of ECM proteins, including collagen and fibronectin, and thereby exacerbate diabetic nephropathy.

Our studies have some limitations. First, we have used a model of type 1 diabetes mellitus rather than more common type 2 diabetes mellitus. Second, type 2 diabetes mellitus patients with diabetic nephropathy often have only partially reduced ADAMTS13 activity rather than severe ADAMTS13 deficiency.10,11,14 This study did not define whether partially reduced ADAMTS13 levels contribute to diabetic nephropathy exacerbation. Future studies will be required to determine the threshold of ADAMTS13-specific activity required to prevent exacerbated diabetic nephropathy. Despite this limitation, in our opinion, the results from this study may have clinical implications. First, our data suggest that ADAMTS13–VWF imbalance may causally contribute to thrombotic angiopathy in diabetic patients rather than simply serving as a biomarker of endothelial dysfunction and oxidative stress. Second, population-based studies have shown a lower efficacy for aspirin in preventing cardiovascular events in patients diabetes mellitus compared with non-diabetic patients.28,29 It is possible that the lower efficacy could be, in part, related to the inability of aspirin to inhibit the formation of ULVWF-rich thrombi in diabetic patients. The use of recombinant ADAMTS13 or N-acetyl cysteine, which is known to reduce hyperactive ULVWF multimers to less active VWF multimers, combined with angiotensin-converting enzyme inhibitors may be helpful in preventing cardiovascular events in diabetic patients.

In summary, for the first time to our knowledge, we show that severe ADAMTS13 deficiency exacerbates diabetic nephropathy, likely mediated by VWF-dependent increased intrarenal thrombosis. Alteration in ADAMTS13–VWF balance may be one of the key pathophysiological mechanisms of increased thrombotic angiopathies observed in diabetic patients.

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**Disclosures**

None.

**References**


**Highlights**

- ADAMTS13 (a disintegrin and metalloprotease with thrombospondin type I repeats-13) is a plasma protease that cleaves prothrombotic ultra-large von Willebrand factor (VWF) multimers, into smaller, less active VWF multimers.
- Clinical studies have shown association between ultra-large VWF multimers and reduced ADAMTS13-specific activity with thrombotic angiopathies in patients with diabetic nephropathy.
- We provide genetic evidence on the causal role of ADAMTS13–VWF axis in diabetic nephropathy exacerbation.
- Alteration in ADAMTS13–VWF balance may be one of the key pathophysiological mechanisms of thrombotic angiopathy in diabetes mellitus.
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Online Supplementary Figures

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Article: Regular

Running title: ADAMTS13/VWF axis in diabetic nephropathy
Supplementary Figure I. Comparison of blood glucose and body weight in non-diabetic and diabetic mice. A. Blood glucose. B. Body weight. N=5-12 mice/group.
Supplementary Figure II. Diabetes incidence was comparable among WT, Adamts13\(^{-/-}\) and Adamts13\(^{-/-}\) Vwf\(^{-/-}\) mice. Mice were made diabetic by injecting multiple low doses of streptozotocin (60 mg/kg, intraperitoneal for five consecutive days). Mice having blood glucose levels above 300 mg/dL (on 2 consecutive days) were considered diabetic and included in the study. N=8-12 mice/group.
Supplementary Figure III. Comparison of blood pressure in non diabetic and diabetic mice. Blood pressure in mice was determined by using a Visitec Systems BP-2000 tail-cuff blood pressure monitoring system as described in materials and methods. N= 5-8 mice/group. NS: non significant.
**Supplementary Figure IV.** Comparison of PAI-1 mRNA levels in frozen perfused kidney extracts of non diabetic and diabetic mice. PAI-1 mRNA was comparable in non-diabetic WT, *Adamts13*<sup>−/−</sup> and *Adamts13*<sup>−/−</sup> *VWF*<sup>−/−</sup> mice. Streptozotocin-induced diabetes significantly increased PAI-1 mRNA expression in kidney homogenates of diabetic mice when compared to non-diabetic mice in all the groups. However, *Adamts13*<sup>−/−</sup> diabetic mice exhibited significant increased PAI-1 mRNA expression when compared to WT diabetic or *Adamts13*<sup>−/−</sup> *VWF*<sup>−/−</sup> diabetic mice. PAI-1 mRNA was normalized with the GAPDH expression level. N=5-6 mice/group. NS: non significant.
Supplementary Figure V. Comparison of Inflammatory cytokines TNFα and IL-1β in perfused kidney homogenates from non-diabetic and diabetic mice. Streptozotocin-induced diabetes increased IL1β and TNFα levels in diabetic mice when compared to non-diabetic mice in all groups. IL1β and TNFα levels were comparable in kidney extracts of WT diabetic, Adamts13−/− diabetic and Adamts13−/− Vwf−/− diabetic mice. N=5-6 mice/group. NS: non significant.
Online Supplementary Material and Methods

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Article: Regular

Running title: ADAMTS13/VWF axis in diabetic nephropathy

Animals
Male, 6–8 weeks old Admts13+/−,1 and Admts13+/−Vwf−/−2 used in this study were on C57BL/6J background (backcrossed more than 15 times). Wild-type (WT) mice on C57BL/6J background were maintained as separate line. The University of Iowa Animal Care and Use Committee approved all procedures and studies were performed according to the current Animal Research: Reporting of In Vivo Experiment guidelines (http://www.nc3rs.org/ARRIVE).

Diabetes induction
Mice were made diabetic by injecting multiple low doses of streptozotocin (60 mg/kg, intraperitoneal for five consecutive days). Successful diabetes induction was tested after 2 weeks by measuring blood glucose. Mice having blood glucose levels above 300 mg/dL (on 2 consecutive days) were included in the study. Controls were nondiabetic mice treated with citrate buffer. To control severe hyperglycemia, all the diabetic mice were injected with Insulin (1U/kg) from Day-14 to Day-21 of diabetes induction. Mice were observed twice a week up to 28 weeks. Bedding material changes were made, whenever required.

Noninvasive blood pressure measurement by tail cuff manometry
Blood pressure in mice was determined by using a Visitech Systems BP-2000 tail-cuff blood pressure monitoring system. Briefly, animals were acclimated to warmed restraint boxes daily for 1 wk. Once acclimated, 30 measurements of systolic blood pressure were averaged from each animal daily for 3 days to assess blood pressure.

Plasma ADAMTS13 specific activity
ADAMTS13 specific activity was calculated by dividing ADAMTS13 activity (mU/ml) with the total ADAMTS13 protein (ng/ml) in plasma. ADAMTS13 activity was determined by fluorescence resonance energy transfer (FRET) assay using a synthetic 73-amino-acid peptide, FRETS-VWF73 as described.3 Of note, by this assay the detected average WT mouse ADAMTS13 activity is nearly 5 times less (0.22U/ml) compared to human ADAMTS13 (1U/ml). ADAMTS13 protein levels (ng/mL) were determined using

3
commercially available ELISA kit (Novatein Biosciences, USA) as per manufacturer’s instruction.

**Plasma VWF levels**

Plasma VWF levels were determined by sandwich ELISA as described. Briefly, mouse plasma was mixed 1:25 with sample diluent and added to wells pre-coated with 1:2000 diluted rabbit anti-human VWF as primary antibody (Dako, Carpinteria, CA) and blocked with blocking buffer. Samples were incubated for 2 hours at 37°C followed by washing and then further incubated with 1:10,000 diluted HRP-conjugated anti-VWF as secondary antibody (Dako, Carpinteria, CA). The HRPO substrate (TMB soluble) was added and reaction was stopped after 30 min by adding 1N H2SO4. The absorbance was measured at wavelength 450 nm and analyzed (VersaMax Microplate Reader). Plasma from Vwf−/− mice served as a negative control. Commercially available human pooled plasma (George King Bio-Medical, Overland Park, KS) and the wild-type mouse pooled plasma were used to calculate the standard curve, and VWF protein levels in the samples were determined using this standard curve.

**Urine and Plasma Biochemical Parameters**

Urine samples were collected using mouse-sized metabolic cages (Nalgene). Urine albumin, urine creatinine, plasma creatinine were determined by commercially available kits (Crystal Chem., IL). Plasma urea levels were quantified by Urea Assay kit from Abcam.

**Tissue preparation**

Mice were anesthetized with ketamine/xylazine and perfused pericardially with PBS containing heparin. Left kidney was carefully separated from the surrounding tissue, weighed and stored at -80°C until further use. Normalized kidney weight was expressed as mg/g of body weight. Mice were again perfused with 10% neutral buffered formalin, right kidney was isolated and stored in formalin solution for histology.

**Histology**

Histology was performed on 4 µm formalin-fixed kidney sections. Histopathological features of diabetic nephropathy were assessed in a blinded fashion. Sections were stained for Periodic Acid Schiff’s (PAS, for mesangial cell expansion) and picrosirius red staining for collagen and examined under examined under a light microscope (Zeiss) as described. The extent of mesangial cell expansion and collagen accumulation in each glomerulus was subjectively graded on a scale of 0 to 4, as described with Grade 0, normal; Grade 1, PAS/picrosirius positive area up to 25% (minimal); Grade 2, PAS/picrosirius positive area 25–50% (moderate); Grade 3, PAS/picrosirius positive area 50–75% (moderate to severe) and Grade 4, PAS/picrosirius positive area 75–100% (severe). From each mouse 5-20 individual glomeruli were graded for total N=5 for diabetic group and N=3 for control group.

**Immunohistochemistry**

All sections were deparaffinized, rehydrated, and subjected to heat-induced antigen retrieval. Sections were blocked with 5% serum at room temperature (RT), from the
species in which the secondary antibody was raised, in tris-buffered saline. Endogenous peroxidase activity was quenched with 0.1% hydrogen peroxide in methanol for 15 min. Sections were stained for fibronectin using anti-fibronectin antibody (mouse monoclonal, clone FBN11, 1:50, Invitrogen, catalog# MA5-11981), and anti-CD41 antibody (rat monoclonal, 1:100, Abcam, catalog #ab3361). After overnight incubation at 4˚C, slides were washed thrice with PBS for 5 minutes and incubated with biotinylated secondary antibody for 1 hr RT. Slides were then incubated with streptavidin-HRP for 40 minutes RT and washed thrice with PBS for 5 minutes and incubated with DAB substrate for less than 2 minutes until color develops. Slides were washed in tap water for 2 minutes and stained with hematoxylin for 3 minutes, washed in tap water and mounted using aqueous mounting medium and examined under a light microscope (Zeiss). Incubation without primary antibodies and/or with isotype-matched immunoglobulins was used as a negative control for immunostaining. For fibronectin, the area of diaminobenzidine positive staining was measured with and graded as mentioned earlier. For CD41, number of CD41 positive glomeruli was counted and reported as % CD41 positive glomeruli in each group.

**Western blot**

Frozen kidney samples were homogenized in RIPA buffer (25 mM Tris pH 7.4, 150 mM NaCl, 1% NP-40) containing 0.1% SDS and 4% proteinase inhibitor (complete protease inhibitor cocktail, Roche). Cold samples were sonicated for total 20 seconds with the10 second gap. Tissue lysates were centrifuged at 14000×g for 30 min at 4˚C and supernatants were used for the determination of protein content (by Lowry method) and subsequent Western blot analysis. Total lysates were mixed with sample loading buffer (Novex by Life Technologies, USA) and heated at 95˚C for 5 min. 30 µg (for fibrinogen) and 60 µg (for CD41) of total protein was loaded per well in 10% SDS-PAGE gel (40% acrylamide/bis stock, 1.5 M Tris, pH 8.8, 10% ammonium persulfate and tetramethylethylenediamine), electrophoresed and transferred to a polyvinylidene difluoride (PVDF) membrane. After blocking for 60 min with blocking buffer (5% nonfat dry milk, 50 mM TrisHCl pH 7.5, 0.05% Tween-20), membranes were incubated with anti-fibrinogen (1:5000, Acris Antibodies, Catalog# AP00766PU-N) and anti-CD41 (1:1000, GeneTex, Catalog# GTX113758) at 4˚C overnight, followed by appropriate secondary antibody (polyclonal goat anti-rabbit IgG, Dako, catalog #P0448) conjugated to horseradish peroxidase (HRP). Proteins recognized by the antibody were visualized by enhanced chemiluminescence kit (Thermo Scientific, NY, USA) according to manufacturer instructions. All blots were stripped and reanalyzed for the β-actin (anti-beta actin antibody from Abcam, catalog# ab8226) as a loading control. Intensity of the bands was measured by densitometry and quantified using NIH-Image J software.

**ELISA assay for VWF, PAI-1, TNF-α and IL-1β**

Frozen kidney samples were homogenized and sonicated as mentioned earlier. Supernatants from kidney homogenates were used for determination of PAI-1 (Abcam, USA) and TNF-alpha and IL-1beta (R & D systems, USA) with commercially available ELISA kits according to the manufacturer’s instructions. VWF levels in kidney were quantified by sandwich ELISA as described above.
Quantitative reverse transcription (RT) real-time polymerase chain reaction (PCR)

PAI-1 mRNA expression in frozen kidney samples was measured by quantitative RT-PCR (n = 5-6 mice/group). Briefly, total RNA from the frozen kidney samples was isolated by the Trizol method (Ambion™, Thermo scientific, USA) and genomic DNA removed by DNA-free Kit (Ambion). Total RNA (~400 ng) was reverse-transcribed using iScript™ Reverse Transcription Reagent Kit (BIO-RAD, CA, USA). Reaction was run on Veriti 96 Well Thermal Cycler; cycling conditions were 25°C for 5 minutes, 42°C for 30 minutes, and 85°C for 5 minutes. PCR primers and 6-carboxy fluorescein-labeled probes for GAPDH (Mm99999915_g1) and PAI-1 (Mm00435858_m1) were purchased from Thermo Scientific. PCR amplification of the cDNA (100 ng) was performed with the Applied Biosystems 7900HT Fast Real-Time PCR machine (total volume 25 µl) at the University of Iowa Genomics Division. Cycling conditions were 50°C for 2 minutes, 95°C for 10 minutes, and 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Data were analyzed using the comparative threshold cycle (ΔΔCT) method with values normalized to GAPDH.

Statistical analysis

Graph Pad Prism (version 7.0) was used for statistical analysis. Statistical comparisons for parametric data were performed using one way analysis of variance followed by Sidak's multiple comparisons test. For non-parametric data, Kruskal-Wallis test followed by Dunn's multiple comparisons test was used. P<0.05 was considered statistically significant.

References