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Reduced Glomerular Endothelial Thrombomodulin Is Associated with Glomerular Macrophage Infiltration in Diabetic Nephropathy

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The endothelial glycoprotein thrombomodulin regulates coagulation, inflammation, and apoptosis. In diabetic mice, reduced thrombomodulin function results in diabetic nephropathy (DN). Furthermore, thrombomodulin treatment reduces renal inflammation and fibrosis. Herein, thrombomodulin expression was examined in human kidney samples to investigate the possibility of targeting thrombomodulin in patients with DN. Glomerular thrombomodulin was analyzed together with the number of glomerular macrophages in 90 autopsied diabetic cases with DN, 55 autopsied diabetic cases without DN, and 37 autopsied cases without diabetes or kidney disease. Thrombomodulin mRNA was measured in glomeruli microdissected from renal biopsies from patients with DN and nondiabetic controls. Finally, glomerular thrombomodulin was measured in diabetic mice following treatment with the selective endothelin A receptor (ET_AR) blocker, atrasentan. In diabetic patients, glomerular thrombomodulin expression was increased at the mRNA level, but decreased at the protein level, compared with nondiabetic controls. Reduced glomerular thrombomodulin was associated with an increased glomerular influx of macrophages. Blocking the ET_AR with atrasentan restored glomerular thrombomodulin protein levels in diabetic mice to normal levels. The reduction in glomerular thrombomodulin in diabetes likely serves as an early proinflammatory step in the pathogenesis of DN. Thrombomodulin protein may be cleaved under diabetic conditions, leading to a compensatory increase in transcription. The nephroprotective effects of ET_AR antagonists in diabetic patients may be attributed to the restoration of glomerular thrombomodulin. *(Am J Pathol 2021, 191: 829–837; https://doi.org/10.1016/j.ajpath.2021.02.002)*

Diabetic nephropathy (DN) is the leading cause of end-stage renal disease in the Western world,[1] occurring in approximately 40% of patients with diabetes mellitus.[2] Impaired function of the glomerular endothelial cells occurs in the early stages of diabetic kidney damage, with a reduction in the glomerular endothelial glycocalyx and altered expression of endothelial adhesion proteins contributing to inflammation and fibrosis.[3,4] The thrombomodulin/protein C signaling pathway is critical for maintaining glomerular endothelial homeostasis and preventing inflammation.[5] Indeed, animal studies indicate that impaired glomerular thrombomodulin signaling contributes to the pathogenesis of DN.[6]

Encoded by the *THBD* gene, the transmembrane glycoprotein thrombomodulin is expressed primarily by endothelial cells and is a component of the endothelial surface glycocalyx.[7] The thrombomodulin protein contains an N-terminal lectin-like domain, six epidermal growth factor (EGF)–like repeats, a serine/threonine-rich domain, a transmembrane domain, and a cytoplasmic C-terminal domain. Under physiological conditions, thrombomodulin binds circulating thrombin via the EGF-like domain, driving activation of the serine-protease–activated protein C (APC), which exerts cytoprotective effects by regulating coagulation, inflammation, and apoptosis.[8] Renal thrombomodulin

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is down-regulated in a mouse model of DN, leading to the reduced production of APC and resulting in increased glomerular apoptosis, glomerulosclerosis, and albuminuria.[6] In addition, thrombomodulin prevents inflammation via APC-independent pathways; thrombomodulin's lectin-like domain directly interferes with macrophage recruitment by regulating NF-κB activity and endothelial adhesion molecule expression[9,10] and suppresses glomerular complement activation in DN.[11] Moreover, treating diabetic mice with thrombomodulin reduces the development of DN and glomerulosclerosis by suppressing glomerular inflammation.[12] Similar nephroprotective effects of thrombomodulin treatment are reported in animal models of nephrotoxic serum glomerulonephritis and progressive renal fibrosis.[13,14] Thus, impaired glomerular thrombomodulin function plays a central role in the pathogenesis of DN in animal models, and treatment with thrombomodulin shows promising effects with respect to reducing inflammation in the kidney.

To date, no studies have attempted to measure the expression of glomerular thrombomodulin in patients with DN, although some studies have reported that the serum levels of soluble thrombomodulin (ie, the cleaved extracellular domain of thrombomodulin) are increased, whereas serum levels of APC are decreased, in diabetic patients.[15,16] Although these findings may reflect impaired thrombomodulin function at the systemic level, they do not provide any information with respect to thrombomodulin expression and signaling in the glomerular vasculature. Interestingly, elevated thrombomodulin serum levels have been associated with impaired function of the endothelial glycocalyx in patients with chronic kidney disease,[17] suggesting a correlation between thrombomodulin expression and glycocalyx integrity. Moreover, treatment with the endothelin A receptor (ET_AR) antagonist, atrasentan, preserves the glomerular glycocalyx in a mouse model of DN.[18] This finding raises the question of whether ET_AR antagonists, which recently drew attention by improving renal outcome in patients with DN,[19] may also restore glomerular thrombomodulin signaling in DN.

To address this question, glomerular thrombomodulin protein levels were measured in renal autopsy samples obtained from a large cohort of patients with diabetes with and without DN, and the relationship between glomerular thrombomodulin levels and the presence of glomerular macrophages was analyzed. In addition, glomerular *THBD* mRNA was measured in microdissected glomeruli obtained from patients with DN. Finally, thrombomodulin expression was studied in diabetic mice following treatment with atrasentan.

Materials and Methods

Autopsy Cohort

Renal autopsy tissue specimens from a previously described autopsy cohort[20,21] were retrieved from the pathology archives of the Leiden University Medical Center (Leiden, the Netherlands). In brief, autopsy samples were obtained from diabetic patients with histologically confirmed DN ($n = 90$), diabetic patients with no clinical or histologic evidence of DN or renal insufficiency ($n = 55$), and age- and sex-matched nondiabetic controls ($n = 37$). The renal autopsy samples were scored for a diagnosis of DN by two investigators who were blinded with respect to the patients' clinical data, and DN was diagnosed in accordance with the established histopathologic classification for DN.[22]

Biopsy Cohorts

Autopsy tissue findings were validated by examining renal biopsies obtained from patients with histologically confirmed DN ($n = 9$); unaffected region of tumor-nephrectomized samples was used as control ($n = 8$).

A second biopsy cohort, previously documented by Baelde et al,[23] was used to measure glomerular *THBD* mRNA using real-time quantitative PCR. In short, glomeruli were isolated using laser microdissection from fresh-frozen biopsies obtained from diabetic patients with DN ($n = 24$) and from nondiabetic controls ($n = 13$), including cadaver donor kidneys unsuitable for transplantation for technical reasons and the nonaffected part of tumor-nephrectomized samples. All samples were collected and handled in accordance with Dutch national ethics guidelines and in accordance with the Code of Conduct regarding the Proper Secondary Use of Human Tissue.

Mice

The diabetic $apoE^{-/-}$ mouse model used in this study has been described previously.[18] In brief, 6-week−old male $apoE^{-/-}$ mice (Jackson Laboratory, Bar Harbor, ME) were rendered diabetic by i.p. injections of streptozotocin. Mice that developed a blood glucose level ≥20 mmol/L were considered diabetic. Beginning 12 weeks after induction of diabetes, the selective ET_AR antagonist, atrasentan (7.5 mg/kg/day), was added to the drinking water for 4 weeks, after which the mice were sacrificed and renal tissues were collected, fixed, and embedded in paraffin for measuring thrombomodulin expression. To measure *Thbd* mRNA, laser-microdissected glomeruli were obtained from the paraffin-embedded renal tissues. All animal experiments were conducted in accordance with national guidelines for the care and use of experimental animals and were approved by the local Animal Experiment Committee.

Immunohistochemistry and Immunofluorescence

All kidney tissues were divided into sections (4 μm thick). Human tissues were immunostained using the following primary antibodies: mouse anti-human EGF-like extracellular domain of thrombomodulin (1:200; Leica Biosystems, Danvers, MA), mouse anti-human intracellular domain of

thrombomodulin (1:100; Santa Cruz Biotechnology, Dallas, TX), and mouse anti-human CD68 (1:2000; Dako Cytomation, Glostrup, Denmark). Mouse tissues were immunostained using the following primary antibodies: rabbit anti-mouse/rat thrombomodulin (1:2000; Abcam, Cambridge, MA) and rat anti-mouse CD68 (1:15; Abcam). The primary antibodies were visualized using an anti-mouse or anti-rabbit Envision (Dako Cytomation) horseradish peroxidase–conjugated secondary antibody, with diaminobenzidine (Dako Cytomation) as the chromogen. For hematoxylin and eosin staining, a standard protocol was used. Double-label immunofluorescence was performed using the anti-human thrombomodulin and goat anti–von Willebrand factor (1:400; Affinity Biologicals Inc., Ancaster, ON, Canada) antibodies, followed by the appropriate fluorescent secondary antibodies, and *Lycopersicon esculentum* agglutinin–fluorescein isothiocyanate (Sigma-Aldrich, St. Louis, MO), after which the slides were mounted using VectaShield (Vector Laboratories, Burlingame, CA). For each immunostaining experiment, an isotype-matched nonspecific antibody was used as a negative control. To analyze the putative relationship between glomerular thrombomodulin expression, the number of glomerular CD68-positive cells, adjacent kidney sections were immunostained, which allowed measurement of both thrombomodulin and CD68 in the same glomerulus. All kidney sections were stained in a single session for immunohistochemical analysis and scanned using a Philips Ultra-Fast Scanner 1.6 RA (Philips, Eindhoven, The Netherlands).

Staining Analysis

The thrombomodulin-positive area per glomerulus was measured in 25 glomeruli per section using ImageJ software version 1.50i (NIH, Bethesda, MD; http://imagej.nih.gov/ij) and is expressed relative to the total glomerular area. The glomeruli used for these measurements were selected at random, and the experimenters (C.A. and H.B.) were blinded with respect to the groups and outcome.

DN can present with heterogeneous histology; specifically, glomeruli from one patient with DN can have different histopathologic stages of DN. Therefore, the relationship between thrombomodulin expression and the number of CD68-positive cells was evaluated in individual glomeruli measured in a subset of diabetic patients either with or without DN and nondiabetic controls ($n = 10$ per group), selected randomly from the autopsy cohort. Then, the association between thrombomodulin and CD68 was measured by taking into account both between-patient differences and within-patient differences. All examined glomeruli were selected at random.

PCR Data

Total RNA was extracted from microdissected glomeruli using TRIzol reagent (Ambion, Austin, TX). *THBD/Thbd* mRNA was then measured with quantitative real-time PCR using the SYBR Green I master mix (Bio-Rad, Hercules, CA) in a Bio-Rad CFX real-time system. C_T values were normalized to the housekeeping gene *GAPDH/Gapdh*. The following primers pairs were used: human *THBD* forward, 5′-ACATCCTGGACGACGGTTTC-3′ and reverse, 5′-CGCAGAT-GCACTCGAAGGTA-3′; human *GAPDH* forward, 5′-CGACCAC-TTTGTCAAGCTCA-3′ and reverse, 5′-AGGGGTCTACATGGC-AACTG-3′; mouse *Thbd* forward, 5′-TCAATGCGTGGAGCATGAGT-3′ and reverse, 5′-AGGAGCGCACTGTCATCAAA-3′; and mouse *Gapdh* foward, 5′-CTCATGACCACAGTCCATGC-3′ and reverse, 5′-CACATTGGGGGTAGGAACAC-3′.

In Vitro Studies

Human umbilical vascular endothelial cells were cultured in Endothelial Cell Growth Medium-2 media (Lonza, Basel, Switzerland) at 37°C in 5% CO_2. THP-1 cells (ATCC, Manassas, VA) were cultured at 37°C in 5% CO_2 in RPMI 1640 medium (Gibco Laboratories, Gaithersburg, MD) supplemented with 10% fetal bovine serum (Sigma-Aldrich). Human umbilical vascular endothelial cells were plated in 96-well plates and grown to 70% confluency before transfection. Cells were then transfected with siRNA against *THBD* or a nontargeting siRNA using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA) for 8 hours, after which cells were grown to confluency in complete medium. Cells were then treated with 50 ng/mL tumor necrosis factor (TNF)-α or vehicle for 12 hours, after which 150,000 Hoechst-labeled THP-1 cells were added to each well. Cells were incubated for 30 minutes at 37°C under static conditions. Subsequently, human umbilical vascular endothelial cells were washed with phosphate-buffered saline to remove the unbound THP-1 cells. Cells were then fixed in 4% paraformaldehyde for 5 minutes, followed by two washes with phosphate-buffered saline. The number of attached THP-1 cells was measured with an ArrayScan XTI High Content Platform (Thermo Fisher Scientific, Waltham, MA) using a 10× objective; 25 fields of view were measured. This experiment was performed in triplicate.

Statistical Analysis

Continuous variables were compared using the *t*-test or one-way analysis of variance. Categorical variables were compared using the χ^2 test or the Fisher exact test, where appropriate. To analyze differences in glomerular thrombomodulin protein levels between groups in the autopsy cohort, a linear mixed model that takes into account varying numbers of observations (glomeruli) within patients was used. The Pearson correlation coefficient was used to analyze correlations. For the association between glomerular thrombomodulin and the number of CD68-positive cells, linear mixed models with random effects for the individual patients and fixed effects of thrombomodulin and group, and

an interaction term for a possibly different effect of group on the thrombomodulin effect were examined. Differences were considered significant at $P < 0.05$. Unless stated otherwise, summary data in the figures are reported as the means ± SEM.

Results

Clinical and Histologic Characteristics

The autopsy cohort included 90 diabetic patients with histologically confirmed DN (62%) and 55 diabetic patients with no evidence of DN (38%), as well as 37 renal autopsy samples from nondiabetic controls without renal pathology. The clinical and histologic characteristics of these 151 cases are summarized in Supplemental Table S1. The duration of diabetes was significantly higher in the diabetic patients with DN compared with the diabetic cases without DN (16.2 versus 8.5 years, respectively; $P = 0.002$); however, these two patient groups did not differ significantly with respect to age, sex, diabetes type, presence of hypertension, serum creatinine level, estimated glomerular filtration rate, or HbA1c level.

Diabetic Patients Have Reduced Glomerular Thrombomodulin Protein Levels

The glomerular thrombomodulin levels were generally low; however, thrombomodulin was present in the peritubular capillaries of all cases. Furthermore, the level of thrombomodulin protein varied widely between glomeruli measured within individual cases. Costaining renal tissue sections in a nondiabetic control for thrombomodulin and von Willebrand factor (a marker for endothelial cells) confirmed that thrombomodulin is expressed in glomerular endothelial cells (Supplemental Figure S1). Overall, glomerular thrombomodulin expression was lower in the diabetic patients with DN compared with nondiabetic controls (Figure 1); compared to controls, DN was significantly associated with lower glomerular thrombomodulin levels ($P < 0.0001$). Interestingly, diabetic patients without DN had reduced thrombomodulin levels compared to controls ($P < 0.001$). Glomerular thrombomodulin levels were inversely correlated with the amount of glomerular C5b-9 deposits ($r = -0.210$; $P = 0.015$) and with the glomerular C5b-9–positive area ($r = -0.167$; $P = 0.024$). In patients with DN, glomerular thrombomodulin protein was inversely correlated with serum HbA1c ($r = -0.374$; $P = 0.032$), serum cholesterol ($r = -0.353$; $P = 0.038$), and the amount of glomerular C1q deposits ($r = -0.302$; $P = 0.004$). In contrast, glomerular thrombomodulin protein was not associated with other histologic changes, such as DN class, glomerular basement membrane thickness, renal interstitial fibrosis and tubular atrophy, and glomerular fibrin depositions (data not shown), or clinical findings, such as estimated glomerular filtration rate, proteinuria, and blood pressure. These findings indicate that thrombomodulin protein is decreased in glomeruli of diabetic patients and that this decrease in glomerular thrombomodulin precedes the onset of clinically overt DN.

In support of the findings obtained with autopsy tissue, glomerular thrombomodulin protein was decreased in biopsy samples obtained from patients with DN compared with samples obtained from nondiabetic patients who underwent tumor nephrectomy (Supplemental Figure S2).

Glomerular *THBD* mRNA Levels Are Increased in Patients with DN

Next, *THBD* mRNA was measured in glomeruli isolated using laser microdissection from kidney biopsies obtained from diabetic patients with DN ($n = 24$) and nondiabetic controls ($n = 13$). The clinical characteristics are summarized in Supplemental Table S2; the C_T values are

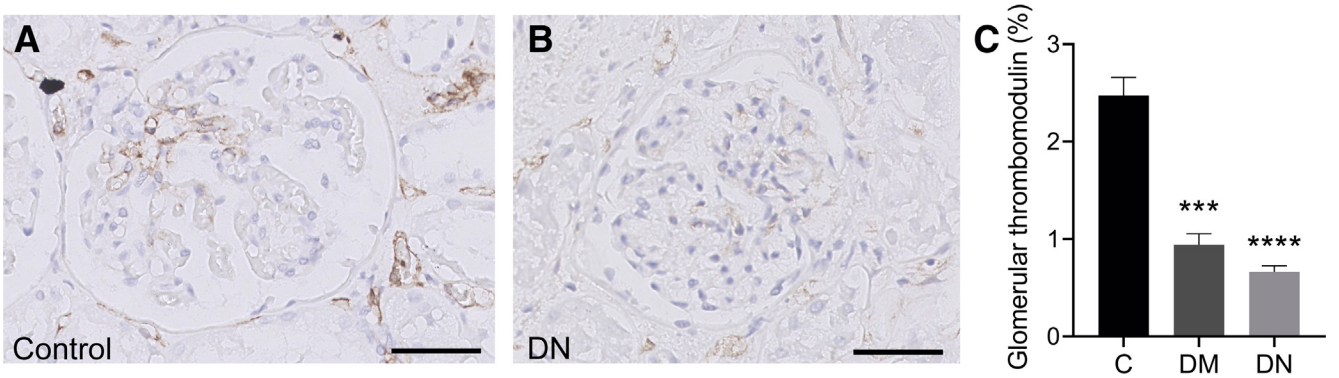

Figure 1 Diabetic patients have reduced glomerular thrombomodulin protein. Immunohistochemical staining using an antibody against the extracellular domain of thrombomodulin in the glomeruli of diabetic patients with diabetic nephropathy (DN), diabetic patients without DN (DM), and nondiabetic controls (C). **A** and **B:** Representative images of thrombomodulin staining in a nondiabetic control (**A**) and a patient with DN (**B**). **C:** Thrombomodulin-positive glomerular area in diabetic patients with DN, diabetic patients without DN, and nondiabetic controls. $n = 90$ diabetic patients with DN (**C: DN**); $n = 55$ diabetic patients without DN (**C: DM**); $n = 37$ nondiabetic controls (**C: C**). ***$P < 0.001$, ****$P < 0.0001$ versus control (linear mixed model). Scale bars = 50 μm (**A** and **B**).

mentioned in Supplemental Table S3. Gene expression analysis revealed that *THBD* mRNA was 2.3-fold higher in the glomeruli of patients with DN compared with that in controls ($P = 0.004$) (Figure 2A), consistent with increased transcription of glomerular *THBD* in diabetic patients with DN. Given the finding of decreased thrombomodulin protein in the glomeruli of diabetic patients, the increased expression of thrombomodulin at the mRNA level may reflect a compensatory transcriptional mechanism in an attempt to offset the reduced endothelial thrombomodulin protein levels in patients with diabetes.

Next, whether thrombomodulin is cleaved in the glomerular endothelium in patients with DN was examined by staining adjacent renal biopsy sections obtained from a patient with DN using antibodies specific to the protein's extracellular and intracellular domains. Staining in the intracellular domain was higher compared with that in the extracellular domain, whereas staining in the surrounding microvessels was similar in the two groups (Figure 2, B and C). This finding, combined with studies reporting increased serum levels of soluble thrombomodulin in patients with DN,[15] indicates that thrombomodulin is cleaved in the glomerular endothelium in DN, thereby releasing the extracellular domain via ectodomain shedding.

Reduced Glomerular Thrombomodulin Expression Is Associated with an Increased Infiltration of Glomerular Macrophages

Next, whether the reduction in glomerular thrombomodulin protein levels is associated with an increase in the number of glomerular macrophages was examined. Similar to the findings with respect to thrombomodulin expression, relatively high interindividual variability with respect to the number of glomerular macrophages was observed.

To compare thrombomodulin and CD68 staining in the same glomeruli, imminohistochemistry was used to measure thrombomodulin and the macrophage marker CD68 in adjacent kidney section obtained from patients. Reduced glomerular thrombomodulin was associated with higher numbers of CD68-positive cells (Figure 3). The slopes and intercepts of the three groups were not significantly different, giving the single regression equation where the patients are allowed to have different intercepts (Figure 3A). The average number of CD68-positive cells in glomeruli that had absent thrombomodulin (<0.1% of the glomerular area) was markedly higher in DN compared with that in diabetic controls and nondiabetic controls (11.5 versus 7.8 versus 7.2 cells, respectively; $P = 0.015$). In addition, sections adjacent to the thrombomodulin- and CD68-stained sections were stained for hematoxylin and eosin. High thrombomodulin and low CD68 staining colocalized in control glomeruli (Figure 3B), and low thrombomodulin and high CD68 staining colocalized in diabetic glomeruli (Figure 3, C and D). However, low thrombomodulin and high CD68 staining did not exclusively colocalize to lesioned areas; for example, the loss of thrombomodulin and the presence of CD68-positive cells are not exclusively localized to the Kimmelstiel-Wilson lesion (Figure 3D). We conclude that glomerular thrombomodulin levels are inversely associated with glomerular infiltration of macrophages, raising the possibility that thrombomodulin may play a role in the migration of macrophages into the diabetic glomerulus.

To support this finding, it was investigated whether reduced endothelial thrombomodulin expression results in an increase in the adhesion of monocytes to activated endothelial cells. Human umbilical vascular endothelial cells were transfected with a thrombomodulin-targeting siRNA or a nontargeting control siRNA, and were activated with TNF-α or vehicle treatment. Then, THP-1 monocytes were applied

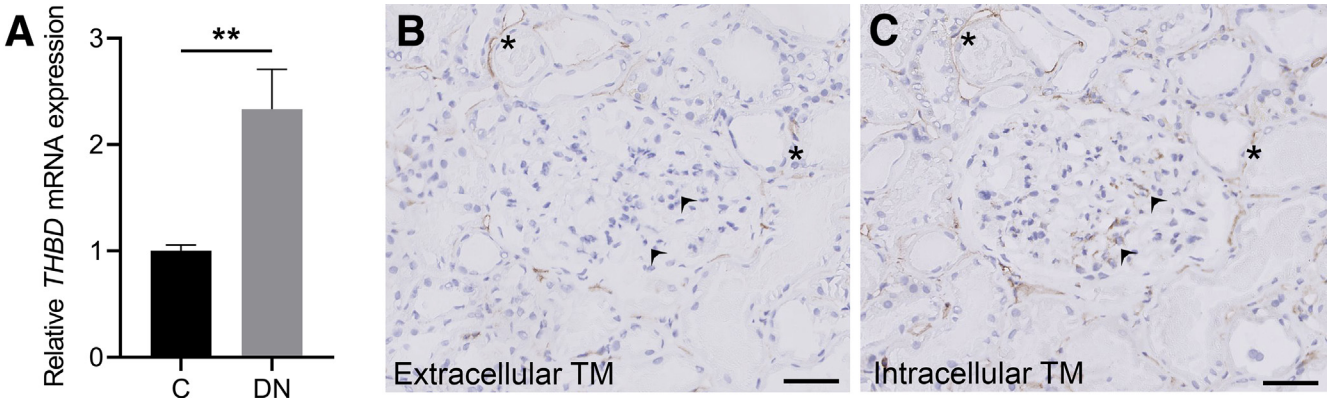

Figure 2 Glomerular *THBD* mRNA is increased in patients with diabetic nephropathy (DN). **A:** Summary of *THBD* mRNA measured in microdissected glomeruli obtained from biopsy samples taken from diabetic patients with DN and nondiabetic controls, expressed relative to control. **B** and **C:** Representative images of adjacent kidney sections obtained from a patient with DN, stained using an antibody against the extracellular (**B**) and intracellular (**C**) domains of thrombomodulin (TM). Note the increased immunoreactivity using the intracellular domain antibody in the same glomerulus (**arrowheads**), with similar immunoreactivity in the surrounding microvessels (**asterisks**). $n = 24$ diabetic patients with DN (**A**); $n = 13$ nondiabetic controls (**A**). $^{**}P < 0.01$ versus control (*t*-test). Scale bars = 50 μm (**B** and **C**).

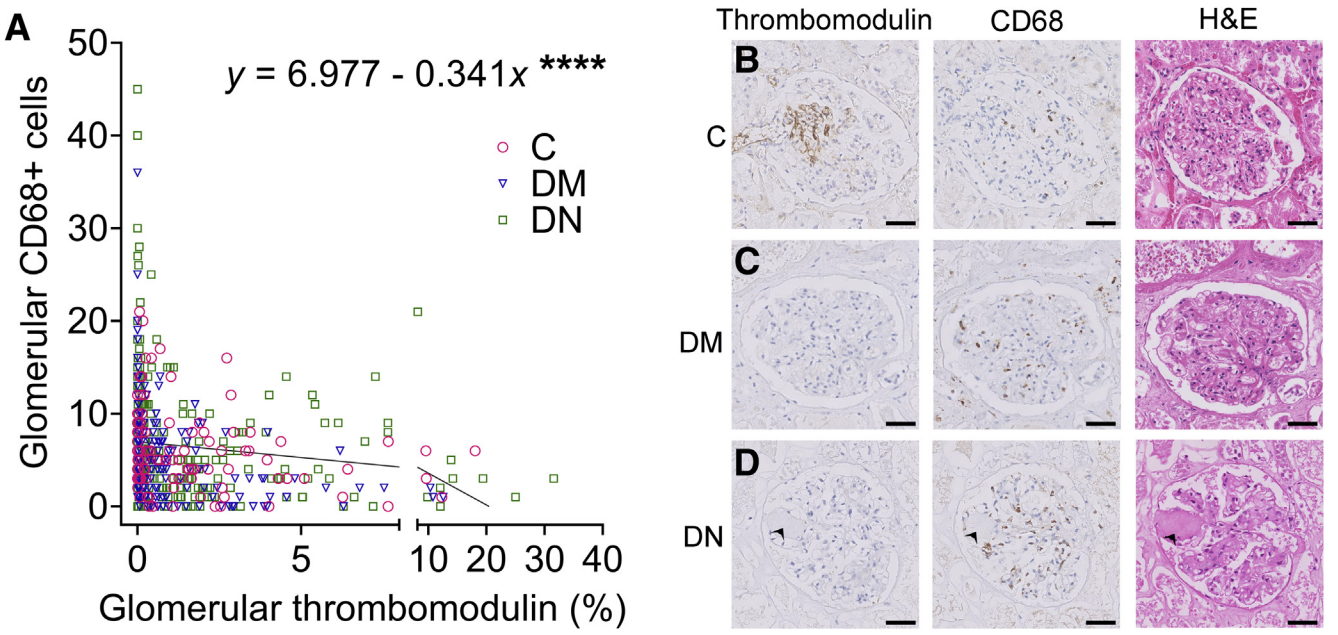

Figure 3 Glomerular thrombomodulin protein levels are inversely correlated with the number of glomerular CD68-positive cells. Glomerular thrombomodulin protein levels and the number of CD68-positive cells were measured in renal autopsy samples from diabetic patients with diabetic nephropathy (DN), diabetic patients without DN (DM), and nondiabetic controls (C). **A:** Linear mixed model regression analysis of the number of CD68-positive cells (on the *y* axis) on the thrombomodulin-positive area (on the *x* axis) in the glomeruli of the indicated groups. Each symbol represents an individual glomerulus. **B**–**D:** Representative images of three sets of adjacent kidney sections showing thrombomodulin, CD68, and hematoxylin and eosin (H&E) staining in the same glomeruli of a nondiabetic control (**B**), a diabetic control without DN (**C**), and a patient with DN (**D**). Kimmelstiel-Wilson lesion (**arrowheads**). $n = 10$ patients per group (**A**). ****$P < 0.0001$ (linear mixed model). Scale bars = 50 μm (**B**–**D**).

for 30 minutes, after which the number of adherent monocytes was counted. In both nontargeting control siRNA and thrombomodulin-targeting siRNA endothelial cells, TNF-α stimulation significantly increased the number of adherent monocytes compared with vehicle ($P < 0.0001$). In the absence of thrombomodulin, TNF-α resulted in a larger increase in adherent monocytes (Supplemental Figure S3). The interaction effect of thrombomodulin expression and TNF-α stimulation on One software monocyte adhesion was significant ($P = 0.028$), meaning that absent thrombomodulin expression augmented the effect of TNF-α on endothelial monocyte adhesion. This indicates that thrombomodulin expression reduces the adhesion of monocytes to endothelial cells activated by TNF-α.

The ET_AR Antagonist, Atrasentan, Increases Glomerular Thrombomodulin Protein Levels in Diabetic Mice

It was next examined whether the reduction in glomerular thrombomodulin is associated with changes in the structural integrity of the glycocalyx (ie, the pericellular matrix). Diabetic *apoE*$^{-/-}$ mice, which have decreased glycocalyx dimensions, were treated with atrasentan, a selective ET_AR antagonist that has been shown to reduce albuminuria and restore the glycocalyx to control (ie, nondiabetic) levels.[18]

Consistent with the findings using human tissues, glomerular thrombomodulin staining was significantly decreased in diabetic mice compared with nondiabetic mice ($P = 0.025$) (Figure 4A); moreover, treating diabetic mice with atrasentan increased the levels of glomerular thrombomodulin nearly to the levels observed in nondiabetic mice ($P = 0.069$) (Figure 4A). At the mRNA level, there was no significant difference in glomerular *Thbd* levels between untreated diabetic mice and controls ($P = 0.186$), and treating diabetic mice with atrasentan had no significant effect on glomerular *Thbd* mRNA levels (Figure 4B). In contrast to the findings in human samples, there was no significant difference in glomerular CD68 staining between control, diabetic, and atrasentan-treated diabetic mice (data not shown). Representative images of glomeruli stained for thrombomodulin and CD68 are shown in Figure 4, C and D, respectively.

Finally, renal tissue sections of a nondiabetic control case and a DN patient were costained with thrombomodulin and lectin, which binds to carbohydrates present in glycocalyx. Thrombomodulin and lectin binding were both present and colocalized in the glomerular endothelium of the nondiabetic control case (Supplemental Figure S4). In glomeruli of the DN patient, thrombomodulin was absent, even though the binding of lectin still demonstrated preservation of glycocalyx constituents on the glomerular endothelial cell surface (Supplemental Figure S4).

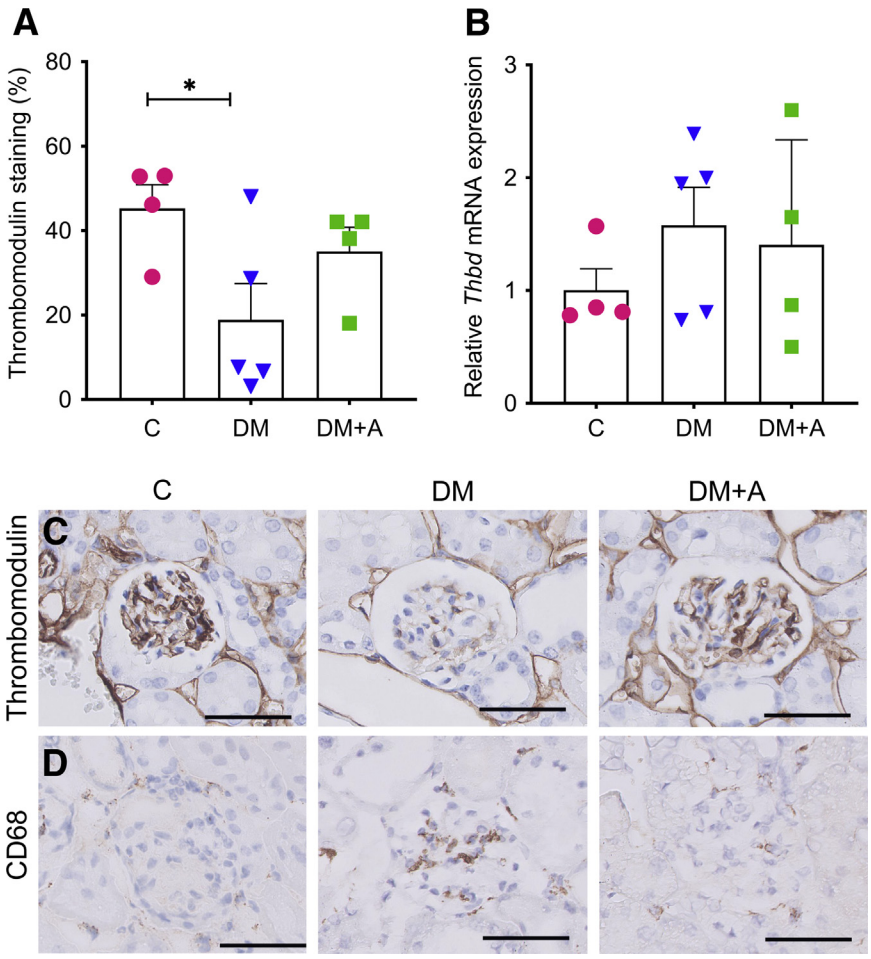

Figure 4 Glomerular thrombomodulin protein levels are restored in diabetic *apoE*$^{-/-}$ mice following treatment with atrasentan. Diabetic *apoE*$^{-/-}$ mice (DM) were treated with the selective endothelin A receptor antagonist, atrasentan (DM + A) (*Materials and Methods*). **A:** Summary of the thrombomodulin-positive area measured in glomeruli stained using an antibody against the extracellular domain of thrombomodulin. **B:** *Thbd* mRNA was measured in microdissected glomeruli obtained from the indicated groups and is expressed relative to the control (C) group. **C** and **D:** Representative images of glomeruli stained using an antibody against the extracellular domain of thrombomodulin (**C**) and an antibody against CD68 (**D**). *$P < 0.05$ (one-way analysis of variance). Scale bars = 50 μm (**C** and **D**).

Discussion

This study shows that in diabetes, glomerular thrombomodulin is decreased at the protein level but increased at the mRNA level, and the reduction in glomerular thrombomodulin protein is associated with an increased infiltration of macrophages in the glomeruli. In addition, blocking the endothelin A receptor restores glomerular thrombomodulin protein levels in a diabetic mouse model. These findings suggest that the reduction in glomerular endothelial thrombomodulin occurs at an early stage of diabetic microvascular damage and contributes to glomerular inflammation in DN.

Current findings in human tissue samples are partially consistent with a previous finding of reduced thrombomodulin expression at both the protein and mRNA levels in the renal cortex of streptozotocin-induced diabetic mice.[6] In contrast, here, there was a significant increase in *THBD* mRNA in the glomeruli of diabetic patients with DN, possibly reflecting a mechanism to compensate for the reduced glomerular thrombomodulin protein levels in these patients. This notion is supported by a previous report that diabetic individuals have significantly increased serum levels of soluble thrombomodulin (ie, the cleaved ectodomain),[13] which suggests that thrombomodulin is proteolytically cleaved in diabetes (eg, via leukocyte-derived proteases,[24] intramembranous rhomboids,[25] and/or other enzymes that remain to be identified).

Interestingly, treating diabetic mice with an ET_AR blocker restored glomerular thrombomodulin levels. Because these mice have an improved glomerular endothelial glycocalyx,[18] we speculate that thrombomodulin may regulate glycocalyx integrity. Blocking glomerular ET_AR signaling may upregulate endothelial thrombomodulin by decreasing hypoxia[26]; the resulting increase in thrombomodulin reduces the glomerular infiltration of macrophages, which produce the heparanase-activating enzyme cathepsin L.[27] The resulting decrease in glomerular cathepsin L may reduce heparanase-mediated degradation of the glycocalyx. In support of this hypothesis, a loss of glomerular thrombomodulin was seen to precede the loss of glycocalyx constituents in DN. The current finding that thrombomodulin levels are restored following treatment with an ET_AR blocker is particularly interesting given that ET_AR blockers significantly improve renal function and reduce proteinuria in patients with DN,[19] possibly by increasing the podocyte number.[28] Thus, the current results suggest that thrombomodulin may play a role in the nephroprotective effects of selective ET_AR blockers.

Macrophages are increasingly recognized as playing a critical role in the pathogenesis and progression of DN. For example, macrophages accumulate in the glomeruli of patients with early-stage DN,[29,30] where they produce cytokines, such as TNF-α and transforming growth factor-β, thereby contributing to glomerulosclerosis by inducing mesangial cells to produce matrix proteins.[31] Furthermore, reducing the glomerular infiltration of macrophages can protect diabetic mice from developing DN.[3,32,33] In this respect, the current results suggest that reduced endothelial thrombomodulin in diabetic patients contributes to glomerular inflammation, even in patients who have not developed DN. Thrombomodulin inhibits macrophage recruitment via several distinct mechanisms. First, the lectin-like ectodomain regulates TNF-induced NF-κB and extracellular signal-regulated kinase 1/2 activation and interferes with high-mobility group box 1 signaling in endothelial cells, thereby decreasing adhesion molecule expression and macrophage extravasation.[9,10,13] Second, thrombomodulin's extracellular EGF-like domain interacts with thrombin, reducing thrombin's chemotactic effects[34] and suppressing NF-κB activity via APC activation.[8] Third, thrombomodulin suppresses the glucose-induced up-regulation of monocyte chemoattractant protein-1 in podocytes.[12] Thus, in diabetes, degradation of the soluble extracellular domains of thrombomodulin in the extracellular milieu leads to a localized reduction in these anti-inflammatory mechanisms. Interestingly, thrombomodulin also has proinflammatory properties. For example, *in vitro* experiments show that the serine-threonine domain of thrombomodulin, which is positioned adjacent to the cell membrane, can increase the binding of macrophages to endothelial cells by binding the integrins lymphocyte function-associated antigen 1 and macrophage-1 antigen (alias integrin αMβ2).[35] Following proteolytic cleavage of thrombomodulin's lectin-like and EGF-like domains, the serine-threonine domain may remain in the cell membrane, where it can promote the glomerular infiltration of macrophages. Thus, the combination of degradation of thrombomodulin's anti-inflammatory extracellular domains in the diabetic glomerulus together with increased exposure of thrombomodulin's proinflammatory domain may promote the infiltration of macrophages into the glomeruli.

The current study has several limitations that warrant discussion. First, autopsy cohorts are inherently limited by the potential incompleteness of clinical data, which may have limited the strength of this analysis with respect to measuring correlations with certain clinical parameters. Also, serum samples of these cases were not available to study the cleaved thrombomodulin levels. Furthermore, the presence of glomerular thrombomodulin in human renal autopsies was generally low, compared with the global thrombomodulin staining pattern observed in mouse glomeruli. The analysis may have been affected by autopsy-related artifacts, such as post-mortem changes in thrombomodulin levels. However, this is unlikely, as the findings obtained using autopsy tissues were supported by results obtained using renal biopsy samples. Furthermore, thrombomodulin was present in the peritubular capillaries of all studied samples. The use of autopsy samples provided several advantages over solely using biopsy-based samples. For example, this approach provided an accurate representation of patients with diabetes mellitus, as renal tissues were analyzed regardless of the duration or severity of diabetes mellitus, the presence of retinopathy, the degree of proteinuria, and estimated glomerular filtration rate. In addition, the use of autopsy material led to studying >25 glomeruli per sample, which is not feasible when using biopsy samples. Finally, the restoration of thrombomodulin levels by atrasentan treatment lacked significance because of a low sample size. Nevertheless, these data are still indicative of an interaction between thrombomodulin and glycocalyx components in DN.

In conclusion, our results demonstrate that the glycoprotein thrombomodulin is significantly reduced in the glomeruli of diabetic patients, regardless of the onset of DN, suggesting that reduced thrombomodulin levels contribute to increased glomerular inflammation in these patients. Furthermore, the reduction in glomerular thrombomodulin protein coincides with a reduction in the glomerular glycocalyx in diabetic mice and is restored by treating diabetic mice with the selective ET_AR blocker atrasentan. Taken together, these findings suggest that impaired thrombomodulin signaling plays a functional role in the development of DN in diabetic patients. Thus, restoring glomerular thrombomodulin levels (eg, using ET_AR blockers) may provide a promising strategy for diabetic patients at risk for developing diabetic nephropathy.

Supplemental Data

Supplemental material for this article can be found at *http://doi.org/10.1016/j.ajpath.2021.02.002*.

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