# Thrombospondin-1 Is an Endogenous Activator of TGF-β in Experimental Diabetic Nephropathy In Vivo

Christoph Daniel,[1] Kathrin Schaub,[1] Kerstin Amann,[2] Jack Lawler,[3] and Christian Hugo[1]

**OBJECTIVE—**Transforming growth factor-β (TGF-β), the central cytokine responsible for the development of diabetic nephropathy, is usually secreted as a latent procytokine complex that has to be activated before it can bind to its receptors. Recent studies by our group demonstrated that thrombospondin-1 (TSP-1) is the major activator of latent TGF-β in experimental glomerulonephritis in the rat, but its role in diabetic nephropathy in vivo is unknown.

**RESEARCH DESIGN AND METHODS—**Type 1 diabetes was induced in wild-type ($n = 27$) and TSP-1–deficient mice ($n = 36$) via streptozotocin injection, and diabetic nephropathy was investigated after 7, 9.5, and 20 weeks. Renal histology, TGF-β activation, matrix accumulation, and inflammation were assessed by immunohistology. Expression of fibronectin and TGF-β was evaluated using real-time PCR. Furthermore, functional parameters were examined.

**RESULTS—**In TSP-1–deficient compared with wild-type mice, the amount of active TGF-β within glomeruli was significantly lower, as indicated by staining with specific antibodies against active TGF-β or the TGF-β signaling molecule phospho-smad2/3 or the typical TGF-β target gene product plasminogen activator inhibitor-1. In contrast, the amount of glomerular total TGF-β remained unchanged. The development of diabetic nephropathy was attenuated in TSP-1–deficient mice as demonstrated by a significant reduction of glomerulosclerosis, glomerular matrix accumulation, podocyte injury, renal infiltration with inflammatory cells, and renal functional parameters.

**CONCLUSIONS—**We conclude that TSP-1 is an important activator of TGF-β in diabetic nephropathy in vivo. TSP-1–blocking therapies may be considered a promising future treatment option for diabetic nephropathy. ***Diabetes* 56:2982–2989, 2007**

According to the World Health Organization, in 2006 the worldwide prevalence of diabetes was 180,000,000 with ongoing increasing incidence (1). Every third diabetic patient develops diabetic nephropathy, which is meanwhile the most common cause of end-stage renal disease in the Western world (2–4), also with an increasing tendency. Diabetic nephropathy is characterized by hypertrophy, increased matrix accumulation and fibrosis, podocyte damage, and thickening of the glomerular basement membrane, leading to renal failure. The key player in the development of pathogenesis in diabetic nephropathy is transforming growth factor-β (TGF-β), as reported in many in vitro and in vivo studies (3,5,6).

TGF-β is secreted as an inactive procytokine complex that consists of the mature, active TGF-β protein noncovalently bound to a dimer of its $NH_2$-terminal propeptide, the so-called latency-associated protein (LAP), and variably to a latent TGF-β binding protein. Before it can bind to its receptors, TGF-β must to be activated extracellularly (7). Exposure of TGF-β to pH changes, γ-irradiation, reactive oxygen species, plasmin, calpain, cathepsin, some integrins, or thrombospondin-1 (TSP-1) activates TGF-β under in vitro conditions (7). The trimeric TSP-1 can activate TGF-β by binding to both the LAP and the mature TGF-β. This complex interaction reportedly leads to a conformational change within the LAP that allows the mature TGF-β protein to bind to its receptors (8,9). There is strong evidence suggesting a potential key role of TSP-1 in TGF-β activation and pathogenesis during diabetic nephropathy. First, induction of TSP-1 by high glucose is reported for different renal cell types such as isolated mesangial cells (10,11), proximal tubule cells (12), and distal tubule cells (13) in vitro. Upregulation of TSP-1 expression by high glucose is regulated by altering cGMP-dependent protein kinase activity (14,15). Second, all of the above-mentioned studies demonstrated that TSP-1 is a major activator of TGF-β in vitro (10–14). Third, upregulation of both TGF-β and TSP-1 was also confirmed during diabetic nephropathy in humans (16), indicating a potential role of TSP-1 for the activation of TGF-β in human diabetic nephropathy. Furthermore, our group demonstrated that TSP-1 is the major activator of TGF-β in an experimental mesangial proliferative glomerulonephritis in rat by two independent methods (17,18).

Nevertheless, the role of TSP-1 in experimental diabetic nephropathy in vivo is still unknown. Therefore, we hypothesized that TSP-1 is an endogenous activator of TGF-β in diabetic nephropathy and investigated whether TSP-1 deficiency is able to suppress activation and therefore function of TGF-β in streptozotozin (STZ)-induced diabetic nephropathy in the mouse.

## RESEARCH DESIGN AND METHODS

The animal studies were performed in accordance with the internal animal review board (Regierung von Mittelfranken: 621-2531.31-17/05). The animals were housed in a room maintained at 22 ± 2°C and exposed to a 12-h dark/12-h light cycle and fed standard mouse chow (Altromin 1324; Spezialfutterwerke, Lage, Germany) and tap water ad libitum. This study was performed in two different background strains comparing TSP-1–deficient with wild-type mice as previously described (19) to robustly test our hypoth-

From the [1]Department of Nephrology and Hypertension, University Erlangen-Nürnberg, Erlangen, Germany; the [2]Department of Pathology, University Erlangen-Nürnberg, Erlangen, Germany; the [3]Division of Cancer Biology and Angiogenesis, Department of Pathology, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, Massachusetts.

Address correspondence and reprint requests to Christoph Daniel, PhD, University Erlangen-Nürnberg, Loschgestr. 8, 91054 Erlangen, Germany. E-mail: christoph.a.daniel@rzmail.uni-erlangen.de.

Received for publication 23 April 2007 and accepted in revised form 12 September 2007.

Published ahead of print at http://diabetes.diabetesjournals.org on 18 September 2007. DOI: 10.2337/db07-0551.

LAP, latency-associated protein; mAb, monoclonal antibody; PAI-1, plasminogen activator inhibitor 1; PAS, periodic acid Schiff; PCNA, proliferating cell nuclear antigen; STZ, streptozotocin; TGF-β, transforming growth factor-β'3b; TSP-1, thrombospondin-1; TUNEL, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick-end labeling.

© 2007 by the American Diabetes Association.

*The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.*

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/56/12/2982/385870/zdb01207002982.pdf by guest on 18 July 2024

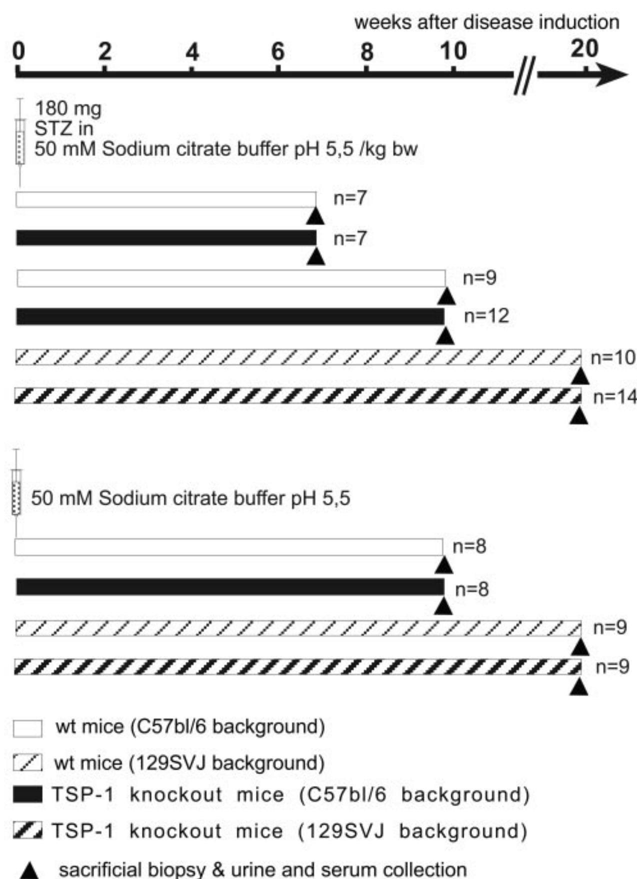


**FIG. 1. Schematic outline of the experimental design.**

esis of TSP-1 as an important mediator of TGF-β activation during experimental diabetic nephropathy. In the first part of the study (7 and 9.5 weeks of diabetes), mice of C57Bl6 background (eight generations backcrossed) were used, and in the second part (20 weeks of diabetes), mice of 129SVJ background were used.

A scheme of the experimental protocol, including group sizes, is shown in Fig. 1. Experimental type 1 diabetes was induced by a single intraperitoneal injection of 180 mg STZ/kg body wt (Sigma, St. Louis, MO) injected in 50 mmol/l sodium citrate buffer (pH 5.5) using 3-month-old mice. Successful diabetes induction was first tested 1 week after induction followed by monthly blood glucose measurements. Nondiabetic control animals were treated with solvent alone. At least seven mice per group with repetitively confirmed high glucose levels (>400 mg/ml) were killed 7, 9.5, and 20 weeks after induction of diabetes. During the long-term (20 weeks) diabetes experiment, we transiently administered long-acting insulin (Lantus, human insulin HI-901; Sanofi Aventis, Frankfurt, Germany) subcutaneously at a dose of 2 units whenever the condition of animals was impaired or weight loss occurred. Using this procedure as described before (20–22), we successfully rendered animals hyperglycemic without becoming ketoacidotic. The day before the mice were killed, a 24-h urine collection was performed and proteinuria, albuminuria, and creatinine clearance were determined. On the day the mice were killed, blood was collected via vena cava puncture, kidneys were perfused with 0.9% sodium chloride followed by perfusion with Deltadex 40 including 0.05% Novocain, and the right kidney was harvested for immunhistochemical analysis. Afterward, the animals underwent perfusion fixation with 48 mmol/l $Na_2HPO_4$ and 14 mmol/l $KH_2PO_4$ including 3% glutaraldehyde for preparation of semi-thin sections (only done for the 20-week diabetes time point).

**Renal morphology and immunohistochemistry.** Tissue for light microscopy was fixed in methyl Carnoy's solution or 4% paraformaldehyde, embedded in paraffin, and cut into 3-μm sections for indirect immunoperoxidase staining, as described previously (23). Sections were also stained with the periodic acid Schiff (PAS) reagent for determination of glomerular matrix expansion. Snap-frozen tissue was cut into 5-μm sections for detection of $CD4^+$ and $CD8a^+$ cells. Glomerular cell number was determined by counting hematoxylin-stained nuclei per 50 glomerular cross-sections within a biopsy. Glomerular hypertrophy was determined by measuring the glomerular tuft area of 60 glomerular cross-sections with computer-assisted morphometry using Metavue software (Visitron, Puchheim, Germany).

To perform immunostaining, tissue sections were incubated with the following primary and secondary antibodies: 19A2, a murine IgG monoclonal antibody (mAb) against the proliferating cell nuclear antigen (PCNA; Chemicon, Temecula, CA) (24), indicating actively proliferating cells; a rat monoclonal IgG2a to mouse CD4 antigen (Caltag Laboratories, Burlingame, CA,); a rat monoclonal IgG2a to mouse CD8a antigen (Caltag Laboratories) (24); F4/80, a murine IgG1 mAb to a surface receptor present on monocytes, macrophages, and dendritic cells (Caltag Laboratories); a polyclonal antibody to human collagen IV (Southern Biotechnology Associates, Birmingham, AL); fibronectin, a rabbit polyclonal antibody to rat fibronectin (Chemicon) (18); a murine $IgG_1$ mAb against TSP-1 (Dunn, Asbach, Germany) (25); a murine mAb for human desmin (DAKO, Glostrup, Denmark) (26); a rabbit polyclonal antibody to phosphorylated Smad2/3 (Santa Cruz Biotechnology, Santa Cruz, CA) (17); a chicken polyclonal anti-human active TGF-β1 (R&D Systems, Wiesbaden, Germany) (18); and a rabbit polyclonal to human plasminogen activator inhibitor-1 (PAI-1; Santa Cruz Biotechnology). Negative controls for immunostaining included either the omission of the primary antibody or substitution of the primary antibody with equivalent concentrations of an irrelevant murine mAb or preimmune rabbit IgG. All immunoperoxidase stainings was done using methyl Carnoy's fixed tissues. After incubation with primary antibodies overnight at 4°C, specific biotinylated secondary antibodies (all by Vector Laboratories, Burlingame, CA) were applied to tissue sections, followed by peroxidase-conjugated Avidin D (Vector Laboratories) and color development with diaminobenzidine, with or without nickel chloride, for nuclear staining.

For frozen sections, a Cy3-labeled donkey anti-rat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) was used as secondary antibody. For each analysis, 25–50 glomerular cross-sections were evaluated in a blinded fashion. Glomerular active TGF-β and expression of fibronectin was quantified by computerized measurement of the positively stained glomerular area using the Metavue Imaging System (Visitron). Mesangial matrix expansion was graded on a scale of 0 to 4 (0, normal/no staining; 1, <25%; 2, 26–50%; 3, 51–75%; and 4, >75% of the glomerulus involved or evaluated in semi-thin sections using a 10 × 10 grid overlaying each glomerulus and counting the number of squares completely filled out by $PAS^+$ mesangium). Collagen IV was graded semiquantitatively and reflected changes in the area and intensity of mesangial staining: 0, very weak or absent staining; 1+, weak staining with <25% of the glomerular tuft showing focally increased staining; 2+, 25–49% of the glomerular tuft with focally increased staining; 3+, 50–75% of the glomerular tuft demonstrating increased staining; and 4+, >75% of the glomerular tuft stained strongly. In addition, glomerular PAI-1 expression was also graded semiquantitatively on a scale of 0 to 4 (0, absent staining; 1, weak podocytic staining; 2, strong podocytic staining; 3, podocytic and mesangial staining; and 4, podocytic and mesangial staining of >50% of the glomerular tuft). For slides immunostained for desmin, only the podocytes at the outer edge of the glomerular tuft were assessed as described elsewhere (26). $F4/80^+$, $CD4^+$, and $CD8a^+$ cells were analyzed in 20 fields (magnification ×400).

**Apoptosis assay.** Apoptotic cells were detected by the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick-end labeling (TUNEL) assay, as described previously (27,28).

**Real-time quantitative RT-PCR.** RNA was isolated from laser microdissected glomeruli using snap-frozen biopsies cut into 10-μm sections, extracted by RLT buffer, and purified using RNeasy columns (both from Qiagen, Hilden, Germany). For each sample, at least 100 laser microdissected glomeruli were used. Subsequently, RNA was treated with DNase to avoid DNA contamination. Reverse transcription was performed using TaqMan reverse transcription reagents (Applied Biosystems, Weiterstadt, Germany) following manufacturer's instructions.

Real-time RT-PCR was performed on a TaqMan ABI 7000 sequence detection system using the Mastermix (all Applied Biosystems). After an initial hold of 3 min at 95°C, samples were cycled 40 times at 95°C for 15 s and 60°C for 60 s. The cDNA content of each sample was compared with 18S (forward primer, 5′- TTGATTAAGTCCCTGCCCTTTGT-3′; reverse primer, 5′- CGATCCGAGGGCCTCACTA-3′) (29) as a housekeeping gene following the ΔΔCt technique. For real-time PCR, the following primers were used: fibronectin (forward primer, 5′-TGTGACCAGCAACACGGTG-3′; reverse primer, 5′-ACAACAGGAGAGTAGGGCGC-3′); TGF-β1 (forward primer, 5′-TGACGTCACTGGAGTTGTACGG-3′; reverse primer, 5′-GGTTCATGTCATGGATGGTGC-3′; probe, 5′-TTCAGCGCTCACTGCTCTTGTGACAG-3′) (30); and TGF-β2 (QuantiTech Primer Assay; Qiagen).

**Miscellaneous measurements.** Urinary protein was measured using the Bio-Rad Protein Assay (Bio-Rad, München, Germany) with BSA (Sigma) as a standard. Creatinine in serum or urine and blood urea nitrogen were measured using an autoanalyzer (Beckman, Munich, Germany). Albuminuria was deter-

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/56/12/2982/385870/zdb01207002982.pdf by guest on 18 July 2024

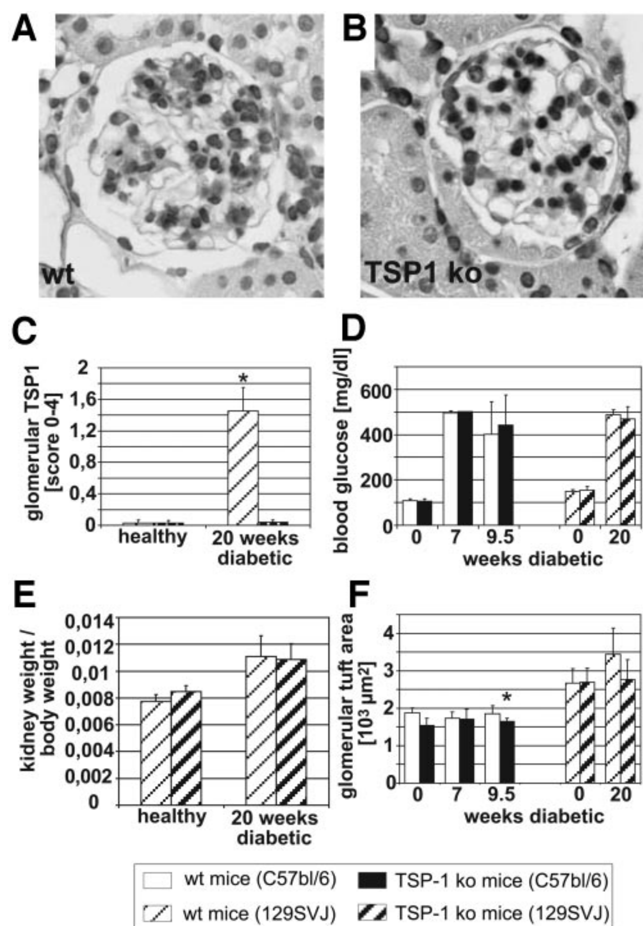


**FIG. 2. TSP-1 is expressed during STZ-induced diabetic nephropathy in mice. Twenty weeks after diabetes induction, TSP-1 is expressed in a typical mesangial cell pattern in wild-type (*A*, cytoplasmatic staining) but not in TSP-1–deficient mice (*B*) as assessed by immunostaining. *C*: TSP-1 immunostaining was quantified 20 weeks after diabetes induction using a semiquantitative score. Induction of diabetes resulted in increased blood glucose levels (*D*), kidney hypertrophy (*E*), and the tendency to enlarged glomeruli (*F*) 20 weeks after induction.**

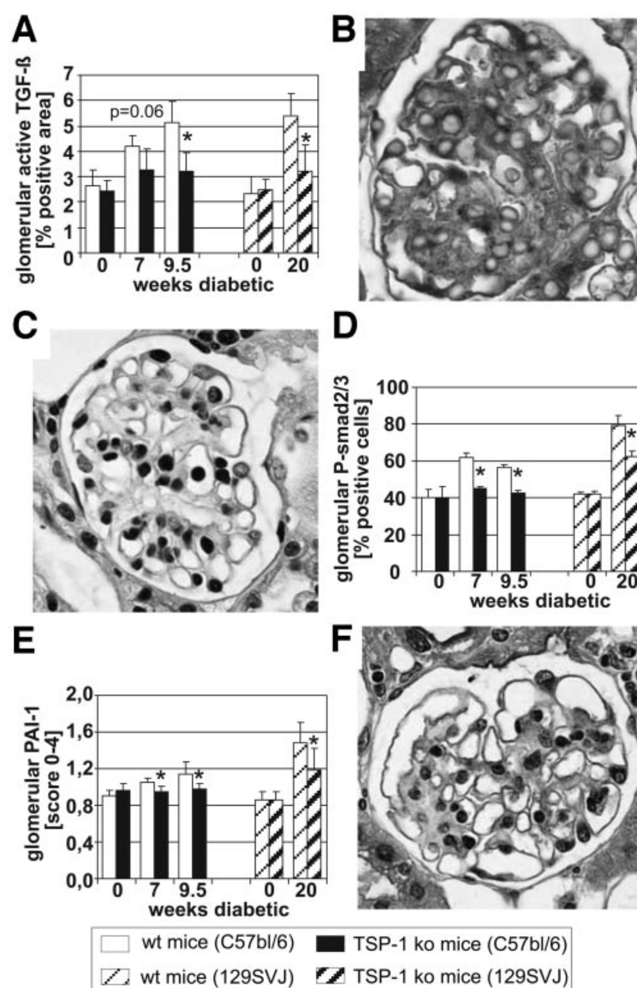


**FIG. 3. TSP-1 deficiency prevents TGF-β activation in diabetic nephropathy. Active TGF-β (*A*; *B*, cytoplasmatic staining), the phosphorylation of the TGF-β signal transduction molecule smad2/3 (*C*, nuclear staining; *D*), and the TGF-β target gene PAI-1 (*E*; *F*, cytoplasmatic staining) were lower during diabetes in TSP-1–deficient mice as shown by immunohistological staining.**

mined using an enzyme-linked immunosorbent assay kit (Axxora, Grünberg, Germany) following the manufacturer's instructions.

**Statistical analysis.** All values are expressed as means ± SD. Statistical significance (defined as $P < 0.05$) was evaluated using the Student's $t$ test or one-way ANOVA with modified $t$ test using the Bonferroni method.

## RESULTS

**TSP-1 is expressed during STZ-induced diabetic nephropathy in mice.** TSP-1 is expressed in diabetic glomeruli of wild-type mice showing a typical mesangial expression pattern 20 weeks after induction of diabetes (Fig. 2*A*) as demonstrated by immunohistological staining (Fig. 2*C*). No staining was observed in glomeruli from TSP-1–deficient mice (Fig. 2*B*) or healthy animals (data not shown). Average blood glucose levels in healthy control animals were detected at ~100 mg/dl and increased to 400–500 mg/dl after induction of diabetes persisting during the whole experiment (Fig. 2*D*). Renal hypertrophy could be seen in all diabetic animals, as demonstrated by an increase of the kidney weight–to–body weight ratio of >40% (Fig. 2*E*). Neither blood glucose levels nor renal hypertrophy was influenced by the lack of TSP-1. Glomerular enlargement during diabetes, as measured by the average glomerular area from cross-sections, was lower in TSP-1–deficient mice compared with wild-type animals, reaching significance after 9.5 weeks but not after 20 weeks ($P = 0.24$) (Fig. 2*F*).

**TSP-1 deficiency reduces TGF-β activation without changing total TGF-β in diabetic nephropathy.** If the de novo expressed TSP-1 is activating TGF-β in STZ-induced diabetic nephropathy, glomerular TGF-β activity but not total TGF-β expression should be reduced in TSP-1–deficient mice. Active TGF-β in glomeruli injured by diabetes was determined by using several different readouts. First, immunostaining using an antibody specifically recognizing the active form of TGF-β1 was performed (Fig. 3*A* and *B*). Second, immunostaining was done with an antibody specific for the phosphorylated form of the TGF-β signal transduction molecules Smad2/3 (Fig. 3*C* and *D*). Finally, staining was done for the TGF-β target gene PAI-1 (Fig. 3*E* and *F*). The amount of active TGF-β steadily increased with duration of diabetes in wild-type mice reaching highest active TGF-β amounts on week 20 (Fig. 3*A*). TSP-1 deficiency was associated with inhibition of increased glomerular TGF-β activity 9.5 and 20 weeks after induction of diabetes (Fig. 3*A*; $P < 0.0005$). Consequently, the increased percentage of glomerular cells positive for the phosphorylated form of the TGF-β signaling molecule Smad2/3 was substantially lower in TSP-1–

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/56/12/2982/385870/zdb01207002982.pdf by guest on 18 July 2024

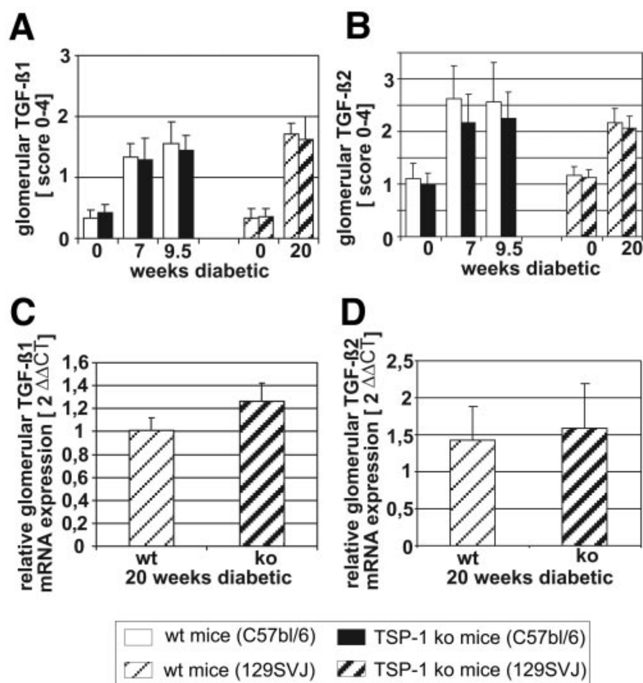


**FIG. 4. TSP-1 deficiency did not change total TGF-β in diabetic nephropathy. Total TGF-β as determined by evaluation of immunostaining of glomerular TGF-β2 (*A*), by real-time PCR for glomerular TGF-β1 on week 20 after diabetes induction (*B*), or by Western blot analysis for cortical TGF-β1 (*C*) or TGF-β2 (*D*) 9.5 weeks after diabetes induction was not changed in TSP-1–deficient mice.**

deficient diabetic mice at all time points investigated (Fig. 3*D*; $P < 0.0005$). TSP-1–deficient mice also showed significantly lower glomerular PAI-1 expression at all time points after diabetes induction (Fig. 3*C*; $P < 0.02$). In contrast, total glomerular TGF-β was similar in wild-type and TSP-1–deficient diabetic mice, as assessed by immunostaining for TGF-β1 and -2 on cross sections (Fig. 4*A* and *B*) and by real-time PCR for TGF-β1 and -2 mRNA from microdissected glomeruli 20 weeks after diabetes induction (Fig. 4*C* and *D*). Western blot analysis for TGF-β1 and TGF-β2 using cortical extracts from mice after 9.5 weeks of diabetes (data not shown) also showed increased levels associated with diabetic nephropathy but no differences in wild-type versus TSP-1–deficient mice.

**Matrix accumulation was reduced in diabetic TSP-1–deficient mice.** Reduction in TGF-β activity was accompanied by reduced glomerular matrix accumulation in diabetic TSP-1 knockout (KO) mice. After 7 weeks of diabetes, glomerular matrix accumulation started, which was much more prominent at week 20 (Fig. 5*A*). Glomerular PAS positivity was significantly lower 7 and 20 weeks after diabetes induction in mice lacking TSP-1 (Fig. 5*A*). Because matrix accumulation was most prominent in mice that were diabetic for 20 weeks, we additionally used semi-thin sections for evaluation of mesangial matrix expansion at this point (Fig. 5*B*). In glomeruli from wild-type mice, mesangial matrix expansion was typically quite prominent 20 weeks after diabetes induction (Fig. 5*B* and *C*). In contrast, the glomeruli from diabetic TSP-1–deficient mice showed minor glomerular changes compared with healthy controls (Fig. 5*D* and *E*) demonstrating a significant reduction of matrix accumulation versus wild-type mice after 20 weeks of diabetes (Fig. 5*B*; $P < 0.02$). Consistent with this result, glomerular fibronectin staining was significantly reduced in TSP-1–deficient diabetic mice at each time point investigated (Fig. 5*F*; $P < 0.003$), and fibronectin mRNA was almost significantly reduced 20 weeks after diabetes induction (Fig. 5*G*; $P = 0.06$). Constitutive expression of glomerular collagen IV in healthy animals only slightly but significantly ($P < 0.03$) increased after diabetes induction in wild-type animals. This increased collagen IV expression during diabetes was prevented in TSP-1–deficient mice 7 and 9.5 weeks after diabetes induction and significantly reduced compared with wild-type animals on week 20 (Fig. 5*H*).

Not only mesangial cells but also podocytes are an important target of renal diabetic injury. Because desmin has been established as an excellent marker of podocyte injury during diabetic nephropathy (26), podocytic desmin expression was evaluated in wild-type and TSP-1–deficient diabetic mice. Podocytic desmin expression was significantly lower in TSP-1–deficient mice 20 weeks after diabetes induction, indicating less podocyte damage in these animals (Fig. 5*I*; $P < 0.05$).

**Influx of inflammatory cells was reduced in diabetic mice lacking TSP-1.** Inflammatory cells are important mediators for the induction of renal fibrosis. Therefore, influx of macrophages and T-cells were investigated in wild-type and TSP-1–deficient mice during STZ-induced diabetic nephropathy. All inflammatory cell types assessed (macrophages via F4/80 staining, T-cells via CD4 and CD8 staining) were increased within the renal cortex during diabetic nephropathy. Accumulation of these different inflammatory cell types within the renal cortex was overall lower in TSP-1–deficient mice, reaching statistical significance at 9.5 weeks (C57Bl6 background) and variably at 20 weeks (129 SVJ background) after diabetes induction. Accumulation of cortical macrophages was significantly lower in diabetic TSP-1–deficient mice at 9.5 but not 20 weeks after diabetes induction because of a high SD (Fig. 6*A*). Cortical influx of T-cells as assessed by CD4 and CD8a positivity was significantly reduced by 52 and 64% in TSP-1–deficient mice 9.5 weeks after diabetes induction compared with wild-type controls (Fig. 6*B* and *C*). In parallel to cortical macrophages, T-cell infiltration was more than four times higher compared with healthy controls in wild-type mice after 20 weeks of diabetes. At that time point, significantly fewer $CD4^+$ cells were again found in TSP-1–deficient mice, but $CD8a^+$ cells only showed a tendency to lower numbers (Fig. 6*B* and *C*) compared with wild-type diabetic mice.

**TSP-1 deficiency reduced glomerular cell number in 20-week diabetic mice.** Because TSP-1 is known to influence both cellular proliferation and apoptosis, these measures were investigated during diabetic nephropathy. During the first 10 weeks of diabetes, glomerular cell number increased only slightly and to an equal extent in wild-type and TSP-1–deficient mice (C57Bl6 background). Twenty weeks after diabetes induction (129SVJ background), a significant increase of glomerular cell number was detected in wild-type mice, which was ameliorated in TSP-1–deficient mice (Fig. 7*A*; $P < 0.0002$). Differences in cell number are due to the balance of local proliferative and apoptotic activity and influx of systemic cell types. Glomerular proliferation was slightly enhanced during diabetes in both groups starting on different levels in the two different backgrounds. Independent of the genetic background, neither the proliferative (Fig. 7*B*) nor the apoptotic activity (as assessed by TUNEL assay; Fig. 7*C*

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/56/12/2982/385870/zdb01207002982.pdf by guest on 18 July 2024

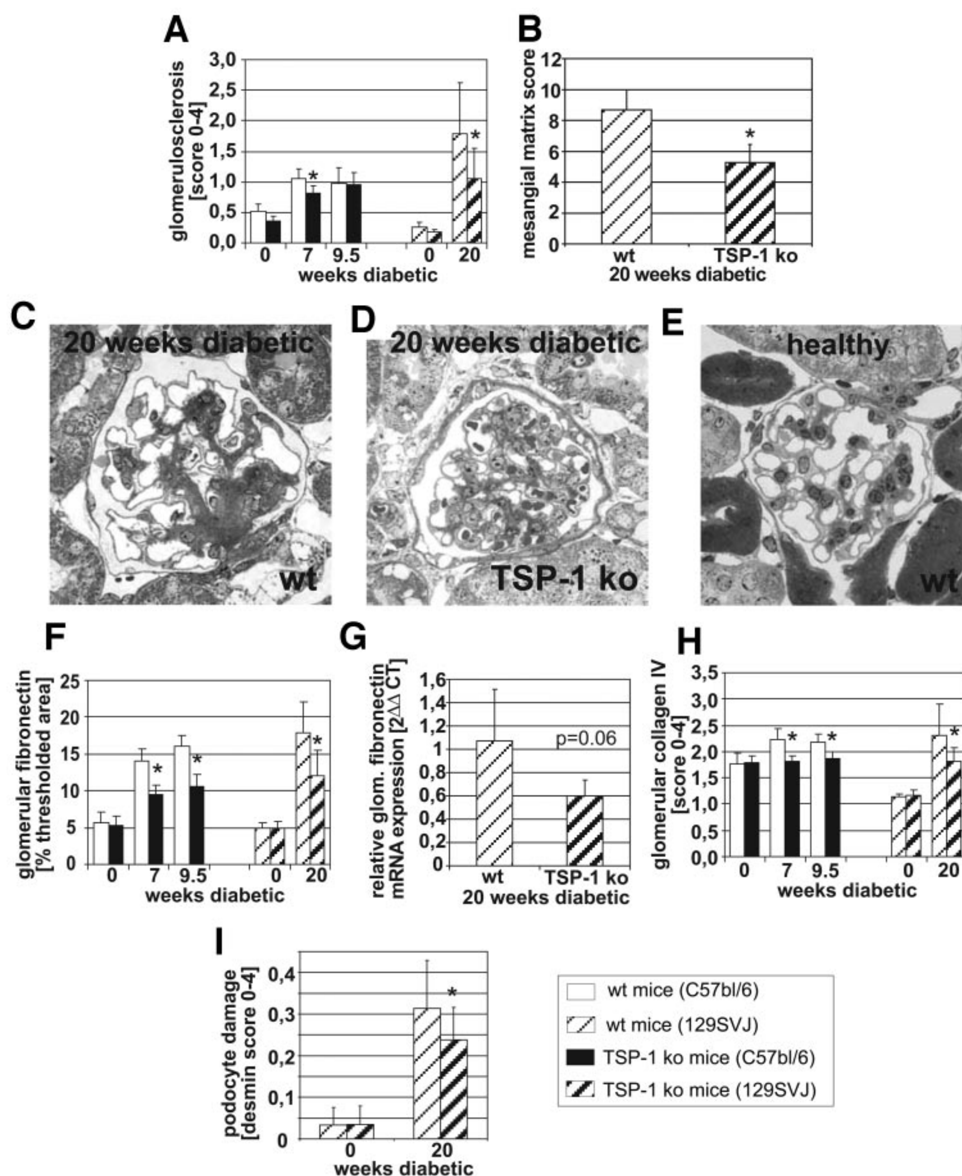


**FIG. 5. Matrix accumulation was lower in diabetic TSP-1–deficient mice. Glomerular matrix expansion was either investigated using PAS staining in all animals (*A*) or using semi-thin sections in week-20 biopsies (*B*). Typical pictures from 20-week diabetic wild-type (*C*) and TSP-1–deficient mice (*D*) and from age-matched healthy controls (*E*) were shown using semi-thin sections. Glomerular matrix expansion was reduced during diabetic nephropathy in TSP-1–deficient mice as assessed by immunohistological staining for fibronectin (*F*) and collagen IV (*H*) or by measuring fibronectin mRNA by real-time PCR (*G*). *I*: Podocyte damage was assessed by immunohistology for podocytic desmin.**

and *D*) within glomeruli or cortical areas was significantly different in TSP-1–deficient versus wild-type mice.

**Kidney function was improved in diabetic mice lacking TSP-1.** Proteinuria, a marker of severity of diabetic nephropathy, was unchanged during early diabetes in both groups, indicating only a minor impairment of renal function at the beginning of diabetic nephropathy. Twenty weeks after diabetes induction, proteinuria in diabetic mice was increased by approximately eightfold compared with healthy controls. This response was decreased by ~50% in TSP-1–deficient mice compared with wild-type animals (Fig. 8*A*; $P < 0.003$). Albuminuria, an even more sensitive marker of renal diabetic injury, was increased during early diabetes in the C57bl6 background (weeks 7–9.5) and was maximally increased 20 weeks after diabetes induction in the 129 SVJ background (Fig. 8*B*). TSP-1–deficient mice showed a tendency to lower albuminuria levels during early diabetes (7–9.5 weeks), which almost reached significance at week 20 (Fig. 8*B*; $P = 0.08$). Serum urea as another indicator of renal functional impairment was also increased during diabetic nephropathy. Serum urea was significantly reduced in TSP-1–deficient mice 9.5 weeks after diabetes induction and showed an almost significant tendency to lower levels 20 weeks after diabetes induction (Fig. 8*C*; $P = 0.06$). Creatinine clearance was the same in both groups (9.12 ± 2.8 in wild-type mice vs. 8.26 ± 3.4 ml/kg body wt in TSP-1–deficient mice), but only the colorimetric and not a more accurate high-performance liquid chromatography–based methodology was used.

## DISCUSSION

Diabetic nephropathy already is the leading cause for end-stage renal disease in the Western world (1,2,4), and the incidence is increasing. Many in vitro and in vivo

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/56/12/2982/385870/zdb01207002982.pdf by guest on 18 July 2024

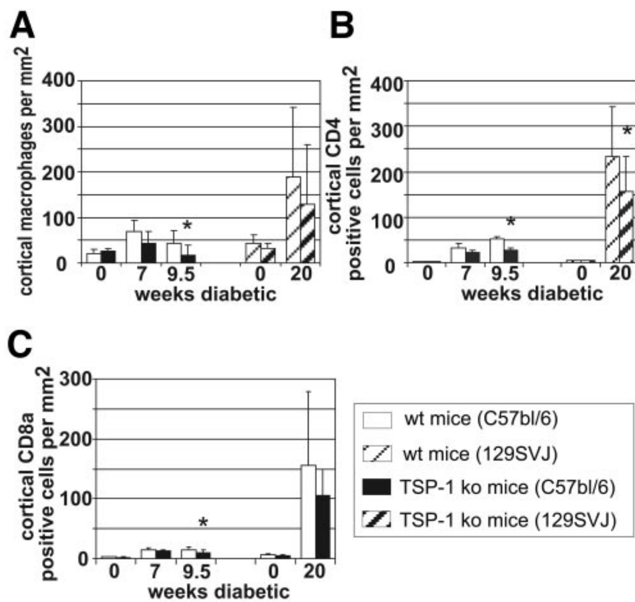


FIG. 6. Influx of inflammatory cells was lower in diabetic mice lacking TSP-1. Influx of cortical macrophages (*A*) and T-cells positive for CD4 (*B*) or CD8a (*C*) were assessed by immunohistological staining.

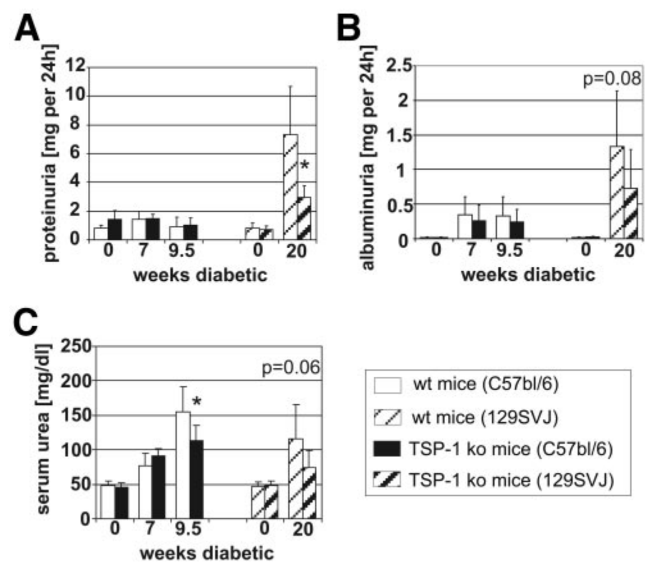


FIG. 8. Kidney function was improved in diabetic mice lacking TSP-1. After diabetes induction, kidney function was assessed by analyzing proteinuria (*A*), albuminuria (*B*), and serum urea (*C*).

studies confirmed TGF-β as a key player in the pathogenesis of diabetic nephropathy (6).

This study, for the first time, shows that TSP-1 is an endogenous activator for TGF-β in diabetic nephropathy in vivo. Deficiency for TSP-1 in diabetic mice reduced activation but not expression of TGF-β in our study. Although glomerular levels of TGF-β1 and TGF-β2 protein and mRNA of diabetic wild-type and TSP-1 KO mice were equivalent, TGF-β activation was reduced in diabetic TSP-1 KO mice, as indicated by several different methods for assessing TGF-β activity directly (specific antibody) or indirectly, via the degree of phosphorylation of the TGF-β signaling molecule smad2/3, as well as the product of the transcriptional TGF-β target, PAI-1. Reduction of TSP-1–mediated TGF-β activation in diabetic nephropathy apparently is able to ameliorate several key features typical of the pathophysiology of diabetic nephropathy, such as increased matrix accumulation and glomerulosclerosis, podocyte injury, glomerular hypertrophy, and proteinuria.

The data presented here suggest that TSP-1 is an important activator of TGF-β in the STZ-induced diabetic nephropathy model. This interpretation is supported by the fact that some diabetic changes (matrix accumulation) in diabetic TSP-1 KO mice are close to healthy controls and the fact that the pattern and the degree of inhibition of diabetic nephropathy features are very similar to studies using general TGF-β–blocking strategies (antibodies) in short-term type 1 (31) or long-term type 2 experimental diabetic nephropathy (32).

Several recent studies demonstrate the importance of inflammation during diabetic nephropathy (33–35). Therefore, the finding of this study that influx of several different inflammatory cell types, such as macrophages and $CD4^+$ and $CD8a^+$ cells, is ameliorated in TSP-1–deficient diabetic mice, compared with wild-type mice suggests that a decreased inflammatory response is the cause of the protective effect of TSP-1 deficiency. It is unclear whether this effect is due to altered active TGF-β levels or to other TSP-1–dependent mechanisms. As discussed in the literature, the role of TGF-β on inflammation is controversial. TGF-β has anti-inflammatory properties as demonstrated by several experimental studies in different models (36,37) and by the fact that TGF-β1–deficient mice die because of multifocal inflammatory disease (38), whereas TGF-β overexpressing mice exert beneficial anti-inflammatory actions of TGF-β (39). On the other hand, TGF-β can induce macrophage chemoattractant protein 1 in mesangial cells (40). TSP-1 itself is known to promote chemo-

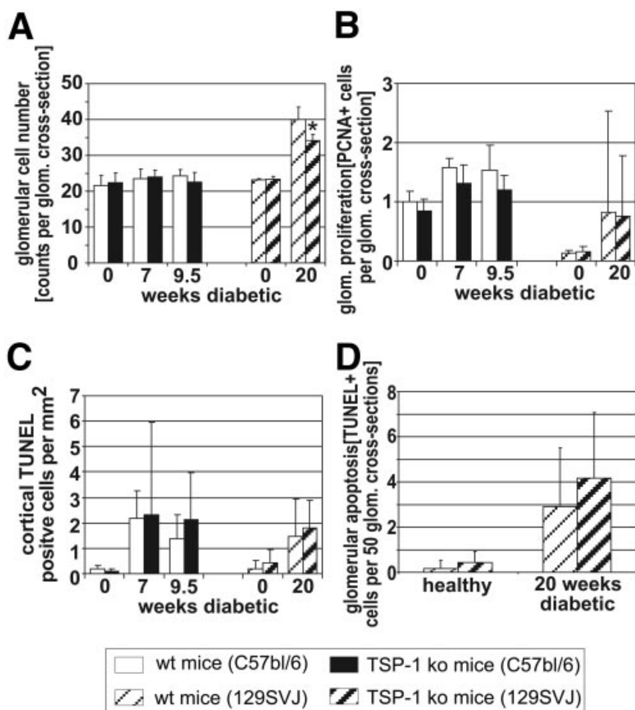


FIG. 7. TSP-1 deficiency reduced glomerular cell number in 20-week diabetic mice. Glomeruli were analyzed for cell number (*A*), and glomerular proliferation was assessed by immunostaining for PCNA (*B*) as a marker for cell proliferation. Apoptotic cells were quantitated either in the cortex during experimental diabetes (*C*) or within the glomerulus on week 20 after diabetes induction (*D*) using TUNEL staining.

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/56/12/2982/385870/zdb01207002982.pdf by guest on 18 July 2024

taxis of leukocytes to inflammatory sites and has a central role in activation and clonal expansion of inflammatory T-cells (41), which would be consistent with the results of our study.

Nevertheless, the limitations of this study need to be discussed. First, participation of other activators of TGF-β or direct secretion of the active cytokine by glomerular cells cannot be completely excluded. Second, after a single high dose of STZ, substantial collateral tissue toxicity may occur, which increases albuminuria (42). TSP-1–mediated effects on destruction/regeneration of β-cells are unlikely, because no differences in blood glucose levels were detected at each time point, but they cannot completely be excluded. Third, our study cannot differentiate whether effects on diabetic nephropathy are due to a systemic, bone marrow–derived, or local lack of TSP-1. Fourth, we show equivalent effects of TSP-1 deficiency in diabetic nephropathy in mice of two different backgrounds, but direct comparison of changes during the time course is invalid because different time points were investigated. Fifth, our STZ-mediated diabetic nephropathy model compared with late diabetic nephropathy in humans showed relatively mild lesions with predominant diabetic glomerulopathy, limiting the conclusions toward human disease. Nevertheless, the 20-week time point of STZ-induced diabetic nephropathy demonstrated clear-cut diabetic glomerular changes that are ameliorated by TSP-1 deficiency. Early treatment or even prophylaxis of diabetic nephropathy in humans would be ideal because TSP-1 expression correlates with disease severity during diabetic nephropathy (16). Furthermore, in humans, only 30% of all diabetic patients develop diabetic nephropathy, suggesting genetic progression factors. In a recent study, peripheral mononuclear blood cells from diabetic patients with and without diabetic nephropathy were screened/compared for candidate target genes using microarray technology. TSP-1 was one out of four differentially regulated genes, suggesting that TSP-1 expression is important for the pathogenesis of diabetic nephropathy in humans (43). In addition, the fact that we could show similar results regarding TSP-1–mediated TGF-β activation and matrix accumulation using mice of two different backgrounds strongly supports the relevance of these findings.

In conclusion, the studies described above identify TSP-1 as an endogenous activator of TGF-β in murine type 1 diabetic nephropathy. Prevention of TSP-1–mediated TGF-β activation in diabetic nephropathy is able to ameliorate several key features typical for the pathophysiology of diabetic nephropathy such as increased matrix accumulation and glomerulosclerosis, podocyte injury, glomerular hypertrophy, and proteinuria. Targeting TSP-1–mediated TGF-β activation is therefore a new therapeutic option to ameliorate diabetic nephropathy.

## ACKNOWLEDGMENTS

This study has received support in part from the Deutsche Forschungsgemeinschaft (SFB 423, TP B6) and from the Interdisciplinary Center for Clinical Research (Project A12).

The technical help of Ulrike Goller, Andrea Braun, Andrea Lüdke, Susanne Weber, and Tanja Christ is gratefully acknowledged.

Parts of this study were presented at the 38th annual meeting of the American Society of Nephrology, Philadelphia, 8–13 November 2005.

## REFERENCES

1. World Health Organization: *Diabetes*: *World Health Organization Fact Sheet*. Geneva, World Health Organization, 2006, p. 1–2 (no. 312)
2. Russell TA: Diabetic nephropathy in patients with type 1 diabetes mellitus. *Nephrol Nurs J* 33:15–28; quiz 29–30, 2006
3. Nicholas SB: Advances in pathogenetic mechanisms of diabetic nephropathy. *Cell Mol Biol (Noisy-le-grand)* 49:1319–1325, 2003
4. Broumand B: Diabetes: changing the fate of diabetics in the dialysis unit. *Blood Purif* 25:39–47, 2007
5. Pantsulaia T: Role of TGF-beta in pathogenesis of diabetic nephropathy. *Georgian Med News* 131:13–18, 2006
6. Ziyadeh FN: Mediators of diabetic renal disease: the case for tgf-beta as the major mediator. *J Am Soc Nephrol* 15 (Suppl. 1):S55–S57, 2004
7. Lawrence DA: Transforming growth factor-beta: a general review. *Eur Cytokine Netw* 7:363–374, 1996
8. Ribeiro SM, Poczatek M, Schultz-Cherry S, Villain M, Murphy-Ullrich JE: The activation sequence of thrombospondin-1 interacts with the latency-associated peptide to regulate activation of latent transforming growth factor-beta. *J Biol Chem* 274:13586–13593, 1999
9. Schultz-Cherry S, Chen H, Mosher DF, Misenheimer TM, Krutzsch HC, Roberts DD, Murphy-Ullrich JE: Regulation of transforming growth factor-beta activation by discrete sequences of thrombospondin 1. *J Biol Chem* 270:7304–7310, 1995
10. Yevdokimova N, Wahab NA, Mason RM: Thrombospondin-1 is the key activator of TGF-beta1 in human mesangial cells exposed to high glucose. *J Am Soc Nephrol* 12:703–712, 2001
11. Poczatek MH, Hugo C, Darley-Usmar V, Murphy-Ullrich JE: Glucose stimulation of transforming growth factor-beta bioactivity in mesangial cells is mediated by thrombospondin-1. *Am J Pathol* 157:1353–1363, 2000
12. Yung S, Lee CY, Zhang Q, Lau SK, Tsang RC, Chan TM: Elevated glucose induction of thrombospondin-1 up-regulates fibronectin synthesis in proximal renal tubular epithelial cells through TGF-beta1 dependent and TGF-beta1 independent pathways. *Nephrol Dial Transplant* 21:1504–1513, 2006
13. Yang YL, Chuang LY, Guh JY, Liu SF, Hung MY, Liao TN, Huang YL: Thrombospondin-1 mediates distal tubule hypertrophy induced by glycated albumin. *Biochem J* 379:89–97, 2004
14. Wang S, Wu X, Lincoln TM, Murphy-Ullrich JE: Expression of constitutively active cGMP-dependent protein kinase prevents glucose stimulation of thrombospondin 1 expression and TGF-β activity. *Diabetes* 52:2144–2150, 2003
15. Wang S, Skorczewski J, Feng X, Mei L, Murphy-Ullrich JE: Glucose up-regulates thrombospondin 1 gene transcription and transforming growth factor-beta activity through antagonism of cGMP-dependent protein kinase repression via upstream stimulatory factor 2. *J Biol Chem* 279:34311–34322, 2004
16. Wahab NA, Schaefer L, Weston BS, Yiannikouris O, Wright A, Babelova A, Schaefer R, Mason RM: Glomerular expression of thrombospondin-1, transforming growth factor beta and connective tissue growth factor at different stages of diabetic nephropathy and their interdependent roles in mesangial response to diabetic stimuli. *Diabetologia* 48:2650–2660, 2005
17. Daniel C, Takabatake Y, Mizui M, Isaka Y, Kawashi H, Rupprecht H, Imai E, Hugo C: Antisense oligonucleotides against thrombospondin-1 inhibit activation of tgf-beta in fibrotic renal disease in the rat in vivo. *Am J Pathol* 163:1185–1192, 2003
18. Daniel C, Wiede J, Krutzsch HC, Ribeiro SM, Roberts DD, Murphy-Ullrich JE, Hugo C: Thrombospondin-1 is a major activator of TGF-beta in fibrotic renal disease in the rat in vivo. *Kidney Int* 65:459–468, 2004
19. Lawler J, Sunday M, Thibert V, Duquette M, George EL, Rayburn H, Hynes RO: Thrombospondin-1 is required for normal murine pulmonary homeostasis and its absence causes pneumonia. *J Clin Invest* 101:982–992, 1998
20. Sharma K, Wang L, Zhu Y, DeGuzman A, Cao GY, Lynn RB, Joseph SK: Renal type I inositol 1,4,5-trisphosphate receptor is reduced in streptozotocin-induced diabetic rats and mice. *Am J Physiol* 276:F54–F61, 1999
21. Taneda S, Pippin JW, Sage EH, Hudkins KL, Takeuchi Y, Couser WG, Alpers CE: Amelioration of diabetic nephropathy in SPARC-null mice. *J Am Soc Nephrol* 14:968–980, 2003
22. Tesch GH, Allen TJ: Rodent models of streptozotocin-induced diabetic nephropathy. *Nephrology* (*Carlton*) 12:261–266, 2007
23. Hugo C, Shankland SJ, Pichler RH, Couser WG, Johnson RJ: Thrombospondin 1 precedes and predicts the development of tubulointerstitial fibrosis in glomerular disease in the rat. *Kidney Int* 53:302–311, 1998
24. Hohenstein B, Kasperek L, Kobelt DJ, Daniel C, Gambaryan S, Renne T, Walter U, Amann KU, Hugo CP: Vasodilator-stimulated phosphoprotein-deficient mice demonstrate increased platelet activation but improved

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/56/12/2982/385870/zdb01207002982.pdf by guest on 18 July 2024

renal endothelial preservation and regeneration in passive nephrotoxic nephritis. *J Am Soc Nephrol* 16:986–996, 2005
25. Hugo C, Pichler R, Meek R, Gordon K, Kyriakides T, Floege J, Bornstein P, Couser WG, Johnson RJ: Thrombospondin 1 is expressed by proliferating mesangial cells and is up-regulated by PDGF and bFGF in vivo. *Kidney Int* 48:1846–1856, 1995
26. Riley SG, Steadman R, Williams JD, Floege J, Phillips AO: Augmentation of kidney injury by basic fibroblast growth factor or platelet-derived growth factor does not induce progressive diabetic nephropathy in the Goto Kakizaki model of non-insulin-dependent diabetes. *J Lab Clin Med* 134: 304–312, 1999
27. Hughes J, Johnson RJ, Mooney A, Hugo C, Gordon K, Savill J: Neutrophil fate in experimental glomerular capillary injury in the rat: emigration exceeds in situ clearance by apoptosis. *Am J Pathol* 150:223–234, 1997
28. Daniel C, Amann K, Hohenstein B, Bornstein P, Hugo C: Thrombospondin 2 functions as an endogenous regulator of angiogenesis and inflammation in experimental glomerulonephritis in mice. *J Am Soc Nephrol* 18:788–798, 2007
29. Hartner A, Schaefer L, Porst M, Cordasic N, Gabriel A, Klanke B, Reinhardt DP, Hilgers KF: Role of fibrillin-1 in hypertensive and diabetic glomerular disease. *Am J Physiol Renal Physiol* 290:F1329–F1336, 2006
30. Overbergh L, Giulietti A, Valckx D, Decallonne R, Bouillon R, Mathieu C: The use of real-time reverse transcriptase PCR for the quantification of cytokine gene expression. *J Biomol Tech* 14:33–43, 2003
31. Sharma K, Jin Y, Guo J, Ziyadeh FN: Neutralization of TGF-β by anti-TGF-β antibody attenuates kidney hypertrophy and the enhanced extracellular matrix gene expression in STZ-induced diabetic mice. *Diabetes* 45:522–530, 1996
32. Ziyadeh FN, Hoffman BB, Han DC, Iglesias-De La Cruz MC, Hong SW, Isono M, Chen S, McGowan TA, Sharma K: Long-term prevention of renal insufficiency, excess matrix gene expression, and glomerular mesangial matrix expansion by treatment with monoclonal antitransforming growth factor-beta antibody in db/db diabetic mice. *Proc Natl Acad Sci U S A* 97:8015–8020, 2000
33. Chow FY, Nikolic-Paterson DJ, Ma FY, Ozols E, Rollins BJ, Tesch GH: Monocyte chemoattractant protein-1-induced tissue inflammation is critical for the development of renal injury but not type 2 diabetes in obese db/db mice. *Diabetologia* 50:471–480, 2007
34. Galkina E, Ley K: Leukocyte recruitment and vascular injury in diabetic nephropathy. *J Am Soc Nephrol* 17:368–377, 2006
35. Mensah-Brown EP, Obineche EN, Galadari S, Chandranath E, Shahin A, Ahmed I, Patel SM, Adem A: Streptozotocin-induced diabetic nephropathy in rats: the role of inflammatory cytokines. *Cytokine* 31:180–190, 2005
36. Kitamura M, Fine LG: Evidence for TGF-beta-mediated 'defense' of the glomerulus: a blackguard molecule rehabilitated? *Exp Nephrol* 6:1–6, 1998
37. Kitamura M: Adoptive transfer of genetically modified macrophages elucidated TGF-beta-mediated 'self-defence' of the glomerulus against local action of macrophages. *Nephrol Dial Transplant* 14 (Suppl. 1):35–38, 1999
38. Shull MM, Ormsby I, Kier AB, Pawlowski S, Diebold RJ, Yin M, Allen R, Sidman C, Proetzel G, Calvin D, Annunziata N, Doetschman T: Targeted disruption of the mouse transforming growth factor-beta 1 gene results in multifocal inflammatory disease. *Nature* 359:693–699, 1992
39. Wang W, Koka V, Lan HY: Transforming growth factor-beta and Smad signalling in kidney diseases. *Nephrology* (*Carlton*) 10:48–56, 2005
40. Wang SN, LaPage J, Hirschberg R: Role of glomerular ultrafiltration of growth factors in progressive interstitial fibrosis in diabetic nephropathy. *Kidney Int* 57:1002–1014, 2000
41. Vallejo AN, Mugge LO, Klimiuk PA, Weyand CM, Goronzy JJ: Central role of thrombospondin-1 in the activation and clonal expansion of inflammatory T cells. *J Immunol* 164:2947–2954, 2000
42. Breyer MD, Bottinger E, Brosius FC III, Coffman TM, Harris RC, Heilig CW, Sharma K: Mouse models of diabetic nephropathy. *J Am Soc Nephrol* 16:27–45, 2005
43. Moczulski DK, Fojcik H, Wielgorecki A, Trautsolt W, Gawlik B, Kosiorz-Gorczynska S, Oczko-Wojciechowska M, Wiench M, Strojek K, Zukowska-Szczechowska E, Grzeszczak W: Expression pattern of genes in peripheral blood mononuclear cells in diabetic nephropathy. *Diabet Med* 24:266–271, 2007

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/56/12/2982/385870/zdb01207002982.pdf by guest on 18 July 2024