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Blockade of TSP1-Dependent TGF- β Activity Reduces Renal Injury and Proteinuria in a Murine Model of Diabetic Nephropathy

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Transforming growth factor- β (TGF- β) is key in the pathogenesis of diabetic nephropathy. Thrombospondin 1 (TSP1) expression is increased in diabetes, and TSP1 regulates latent TGF- β activation *in vitro* and in diabetic animal models. Herein, we investigate the effect of blockade of TSP1-dependent TGF- β activation on progression of renal disease in a mouse model of type 1 diabetes (C57BL/6J-*Ins2^{Akita}*) as a targeted treatment for diabetic nephropathy. Akita and control C57BL/6 mice who underwent uninephrectomy received 15 weeks of thrice-weekly i.p. treatment with 3 or 30 mg/kg LSKL peptide, control SLK peptide, or saline. The effects of systemic LSKL peptide on dermal wound healing was assessed in type 2 diabetic mice (*db/db*). Proteinuria (urinary albumin level and albumin/creatinine ratio) was significantly improved in Akita mice treated with 30 mg/kg LSKL peptide. LSKL treatment reduced urinary TGF- β activity and renal phospho-Smad2/3 levels and improved markers of tubulointerstitial injury (fibronectin) and podocytes (nephrin). However, LSKL did not alter glomerulosclerosis or glomerular structure. LSKL did not increase tumor incidence or inflammation or impair diabetic wound healing. These data suggest that selective targeting of excessive TGF- β activity through blockade of TSP1-dependent TGF- β activation represents a therapeutic strategy for treating diabetic nephropathy that preserves the homeostatic functions of TGF- β . (*Am J Pathol* 2011, 178:2573–2586; DOI: 10.1016/j.ajpath.2011.02.039)

Despite emphasis on tight glycemic control, diabetic complications remain a significant cause of morbidity and mortality and represent a growing economic burden. End-stage renal disease remains a significant cause of mortality and morbidity for diabetic patients and now is the most common

cause of renal failure in the United States.^{1,2} Development of novel targeted therapeutic approaches is needed to serve this growing patient population.

It is well established that transforming growth factor- β (TGF- β) is a key mediator of the cellular processes that induce end-stage renal disease in diabetes, including glomerular hypertrophy with mesangial matrix expansion, renal tubular injury and interstitial fibrosis, and alterations in podocyte slit barrier function, leading to proteinuria.^{1,3–5} There is evidence from cell cultures, animal models, and clinical studies that TGF- β is a primary mediator of the fibrogenic effects of hyperglycemia in diabetic nephropathy.^{6,7} Elevated TGF- β activity also has a role in podocyte dysfunction, including epithelial mesenchymal transition of the podocyte with loss of slit diaphragm proteins associated with the filtration barrier, such as nephrin, and, ultimately, podocyte apoptosis at higher TGF- β concentrations.^{8,9} The diabetic milieu, including hyperglycemia, the renin-angiotensin system, and increased reactive oxygen species, stimulates increased TGF- β activity.¹⁰ Increased expression of TGF- β in glomeruli is documented in diabetic patients and in animal models.^{11–13} Treatment of diabetic mice with neutralizing antibodies to TGF- β reduces mesangial matrix expansion, with differential effects on proteinuria, depending on the specific anti-TGF- β antibody used.^{12,14–16} Thus, TGF- β has been considered as a therapeutic target for the treatment of diabetic nephropathy.¹⁷ However, TGF- β is critical for homeostasis: genetic ablation of TGF- β , its receptors, or its signaling mediators results in

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developmental defects, unremitting inflammation, and an increased incidence of some types of carcinomas.^{18,19} It is not clear to what extent clinical treatment with global TGF- β antagonists would replicate the detrimental effects of genetic deletion of TGF- β or its signaling components. However, given the need for long-term treatment in diabetes, it would be therapeutically advantageous to target only the excessive levels of TGF- β activity induced by diabetic conditions without affecting homeostatic activity.

One of the major control checkpoints for regulating TGF- β activity resides in conversion of the inactive precursor form of TGF- β (latent TGF- β) to the biologically active molecule, a process called activation.²⁰ Activation can be achieved by multiple tissue- and disease-specific mechanisms, including modification of the latent complex by reactive oxygen species, proteolysis, integrin binding, cellular contractility, and shear forces, or through binding to thrombospondin 1 (TSP1).^{21–24} We identified the role of TSP1 as a major physiologic activator of latent TGF- β .^{25,26} Activation occurs by a nonproteolytic mechanism through binding of the type 1 repeats of TSP1 to a conserved sequence [leucine serine lysine leucine (LSKL)] in the latency associated peptide (LAP) region of the latent complex: LAP binding to the mature domain is required to confer latency, and the RFK sequence of TSP1 disrupts this interaction.^{27,28} Peptide mimetics of these sequences can be used to antagonize TSP1-mediated latent TGF- β activation (LSKL and GGWSHW) or, alternatively, to stimulate activation [lysine arginine phenylalanine lysine (KRFL) and RKPK].²⁹

Many factors associated with increased TGF- β activity in diabetes also stimulate TSP1 expression. The increased oxidative stress under high glucose conditions stimulates increased TSP1 protein expression by mesangial cells due to decreased nitric oxide–protein kinase G–mediated transcriptional repression and increased expression of the transcription factor USF2.^{30,31} Glucose also increases TSP1 expression by podocytes,³² and angiotensin II also increases TSP1 expression by renal mesangial cells and cardiac and renal interstitial cells *in vitro*, which results in increased TGF- β activity.^{7,31,33} There is also evidence that TSP1-mediated latent TGF- β activation is involved in the development of diabetic nephropathy: TSP1 protein is increased in the glomeruli of patients with types 1 and 2 diabetic nephropathy, which correlates with increased TGF- β activity.^{34,35} Moreover, streptozotocin-treated TSP1 null mice do not develop glomerulosclerosis or podocyte loss,³⁶ and mice with induced expression of USF2 have increased TSP1 expression, TGF- β activity, and renal fibrosis.³⁷ TSP1 expression correlates with proteinuria in patients with type 2 diabetes,³⁴ and glycated albumin stimulates increased TSP1 expression and tubular hypertrophy.³⁸ Moreover, antagonism of TSP1-dependent TGF- β activation by treatment with a peptide antagonist of TSP1-dependent TGF- β activation, LSKL, reversed myocardial fibrosis and improved left ventricular function by reducing TGF- β activity in diabetic rats.³⁹ Together, these results suggest that the TSP1–TGF- β axis is a key regulator of fibrotic diabetic complications.

Because *in vitro* studies show that the TSP1 antagonist peptides selectively block only the excessive TGF- β ac-

tivity stimulated by either glucose or angiotensin II without affecting basal TGF- β activity, antagonism of TSP1-dependent TGF- β activation represents a targeted approach to reducing the excessive TGF- β activity in the diabetic milieu. In the present studies, we tested whether antagonism of TSP1-dependent TGF- β activation by i.p. injection of LSKL peptide would improve renal function in a mouse model of type 1 diabetes. The Akita C57BL/6J-*Ins2*^{Akita} mouse with unilateral nephrectomy was used.^{40,41} Potential complications of TGF- β antagonism, including inflammation, tumorigenesis, and altered wound healing, were also examined. Akita mice treated for 15 weeks with i.p. injections of LSKL peptide showed decreased proteinuria and fibronectin expression, increased nephrin expression, and reduced TGF- β activity without increases in tumor incidence or inflammation. Systemic administration of LSKL peptide did not impair wound healing in diabetic *db/db* mice. Together, these data suggest that selective targeting of excessive TGF- β activity by blockade of TSP1-dependent TGF- β activation represents a therapeutic target for diabetic nephropathy that preserves the homeostatic functions of TGF- β .

Materials and Methods

Animals

This study was approved by the University of Alabama at Birmingham Institutional Animal Use and Care Committee. Mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Heterozygous C57BL/6J male mice with the Akita mutation (C57BL/6J-*Ins2*^{Akita}) and male wild-type mice (C57BL/6J) were used for the nephropathy studies. Male diabetic *db/db* mice (BKS.Cg-*m* +/+ *Lepr*^{db}/J) were used for skin-wounding studies. Mice were 10 to 12 weeks old when the studies were initiated.

Antibodies and Peptides

Antibodies were purchased from the following vendors: mouse monoclonal anti-TGF- β (pan-specific) neutralizing antibody (clone 1D11) from R&D Systems (Minneapolis, MN), rabbit anti-phospho-Smad2 (Ser465/467) from Cell Signaling Technology Inc. (Beverly, MA) for immunohistochemistry (IHC) analysis and Western blot analysis, goat anti-phospho-Smad2/3 (Ser423/425) and goat anti-Smad2/3 (N-19) from Santa Cruz Biotechnology (Santa Cruz, CA) for Western blot analysis, murine monoclonal anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from Abcam Inc. (Cambridge, MA), rabbit anti-rat fibronectin used for IHC analysis (Life Technologies, Carlsbad, CA), mouse monoclonal anti-ED-A fibronectin (IST-9; Abcam Inc.) for Western blot analysis; rabbit polyclonal to anti-nephrin (Abcam Inc.), and nonimmune mouse IgG from Sigma-Aldrich Corp. (St. Louis, MO). Rat anti-mouse F4/80 antigen IgG (MCA497GA) was purchased from AbD Serotec (Raleigh, NC).

LSKL and SLLK peptides were synthesized by AnaSpec Inc. (San Jose, CA) and were purified by reversed-phase high-performance liquid chromatography with >95% purity determined by mass spectrometry. The lyophilized

peptides were stored at -20°C and were resuspended in saline or PBS before each use.

Uninephrectomy Model

All Akita and wild-type mice underwent a left nephrectomy at 11 to 13 weeks of age. Mice were anesthetized with isoflurane. Under sterile conditions, a longitudinal incision was made in the left flank, the kidney was isolated, and two ligatures were placed around the renal pedicle using 4–0 silk sutures (Ethicon Inc., Somerville, NJ). The pedicle was cut between the two ligatures to remove the left kidney. The surgical wound was closed using 5–0 polypropylene sutures (Ethicon Inc.).

Peptide Administration

Sterile solutions of LSKL or SLLK peptide were made in stock solutions of 3.0 mg/mL (high dose) or 0.3 mg/mL (low dose) in sterile saline. The i.p. injection of LSKL, SLLK, or saline began 2 weeks after uninephrectomy and continued thrice weekly for 15 weeks. For the low-dosage treatment regimen, each group of 20 mice received 3 mg/kg body weight of peptide (LSKL or SLLK) per injection or saline (100 μL /10 g body weight per injection). For the high-dosage treatment regimen, Akita mice were given i.p. injections of LSKL or SLLK peptide at 30 mg/kg body weight per injection or saline (100 μL /10 g body weight per injection).

Body Weight, Blood Pressure, and Blood Glucose Measurements

Body weight was measured using an electronic top-loading balance. Systolic blood pressure was measured using a computerized tail-cuff system (Hatteras Instruments Inc., Cary, NC) in awake, trained mice at ambient room temperature. Blood pressure values were taken at 0, 8, and 15 weeks of treatment for high-dose Akita mice and at 15 weeks for low-dose Akita and BL/6 mice. Six measurements were taken for each mouse. Blood glucose level was measured after a 6-hour fast at 0 weeks (2 weeks after nephrectomy and before the start of treatments) and after 8 and 15 weeks of peptide treatment. Blood samples were collected via the lateral saphenous vein using a 23-gauge needle. Glucose level was measured using an Ascensia Contour glucometer (Bayer Healthcare LLC, Mishawaka, IN).

Analysis of Kidney Function

Twenty-four-hour urine samples were collected during week 15 of treatment from mice housed individually in metabolic cages (Thermo Fisher Scientific Inc., Rochester, NY) with free access to water and food. Urine samples were centrifuged for 10 minutes at 4°C , and the supernatant was stored at -80°C until use. Urine albumin concentration was measured using a competitive enzyme-linked immunosorbent assay method using a 1:3 dilution of urines (Albuwell M kit; Exocell Inc., Philadel-

phia, PA) and was normalized to body weight and urine volume. Urine creatinine level was measured in samples diluted 1:5 using the picric acid method (Creatinine Companion kit; Exocell Inc.) following the manufacturer's instructions. Serum creatinine levels were measured by liquid chromatography–mass spectrometry at the University of Alabama at Birmingham–University of California at San Diego O'Brien Core Center for Acute Kidney Injury.

Plasminogen Activator Inhibitor-1 Luciferase Assay for Detecting TGF- β Activity

Active TGF- β in the mouse urine was quantified using the plasminogen activator inhibitor-1 luciferase reporter assay in mink lung epithelial cells stably transfected with the TGF- β response element of the human plasminogen activator inhibitor-1 gene promoter fused to the luciferase reporter gene construct (a gift from D.B. Rifkin, New York Medical Center, New York, NY).⁴² Mink lung epithelial cells were plated in 24-well plates at a density of 2×10^5 cells per well with incubation medium (4.5 g/mL glucose,⁴³ Dulbecco's modified Eagle's medium + 10% fetal bovine serum, 2 mmol/L L-glutamine, and 200 μg /mL G418 sulfate). Cells were allowed to attach for 4 hours at 37°C and then were washed with serum-free medium. Urine was diluted with serum-free medium at a 1:3 ratio for most samples. The urine was then adjusted to approximately pH 7.0 using 0.1N NaOH. Diluted urine was added to the mink lung epithelial cells in 24-well plates at 0.5 mL per well and was incubated for 16 hours at 37°C at 5% CO_2 . After washing, mink lung epithelial cells were lysed and prepared using a luciferase assay system (Promega Corp., Madison, WI). Luciferase activity in relative light units was read using an Orion microplate luminometer (Berthold, Pforzheim, Germany) and was converted to TGF- β activity (picomoles) by plotting against a standard curve generated by human recombinant TGF- β 1 (R&D Systems). All the samples were assayed in triplicate, and each assay was repeated on three different occasions.

Western Blot Analysis

Renal tissues were homogenized in ice-cold radioimmunoprecipitation assay buffer [25 mmol/L Tris-HCl (pH 7.6), 150 mmol/L NaCl, 1% Nonidet P-40 (Caledon Laboratories Ltd., Georgetown, ON, Canada), 1% sodium deoxycholate, and 0.1% SDS] containing protease inhibitor and phosphatase inhibitor (Sigma-Aldrich Corp.). The cell lysates were centrifuged at 14,000 rpm for 30 minutes at 4°C . The supernatant was collected and used as renal lysates. The protein concentration of lysates was determined by using a Pierce BCA protein assay kit (Thermo Fisher Scientific Inc., Waltham, MA). Samples were mixed with equal volumes of SDS reducing buffer (0.5 mol/L Tris-HCl, pH 6.8, 10% SDS, 25% glycerol, 0.5% bromophenol blue, and 0.05% mercaptoethanol added before use), boiled for 5 minutes, and applied to SDS-PAGE. The proteins were transferred to an Immobilon-P transfer membrane (Millipore, Bedford, MA) and then were blocked in

Tris-buffered saline with 0.1% Tween 20 (Roche Diagnostics GmbH, Mannheim, Germany) containing 5% bovine serum albumin. The membranes were probed with the following primary antibodies overnight at 4°C: anti-phospho-Smad2 (Ser465/467) (1:500), anti-phospho-Smad2/3 (Ser423/425) (1:1000), antinephrin (1:2000), and anti-ED-A fibronectin (1:2000). Anti-GAPDH (1:100,000) was used as a measure of protein loading. After washing, membrane blots were incubated with appropriate secondary antibodies conjugated with peroxidase (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) for 1 hour at room temperature. Immunoreactive bands were detected by enhanced chemiluminescence (PerkinElmer Life Sciences Inc., Boston, MA). Band density was quantified using ONE-Dscan gel image analysis software.

Skin Punch Biopsy Wounding Model

The skin punch biopsy wounding model was conducted as described previously.⁴⁴ Mice male (BKS.Cg-*m* *+/+* *Lepr^{db}/J*), (*db/db*) at approximately 10 weeks of age were anesthetized, the skin was cleaned, and hair on the dorsal skin was removed. Under sterile conditions, two 4-mm-diameter circular, full-thickness wounds, 2 cm apart, were generated on the back of each mouse at the same craniocaudal level using a dermal punch (Acuderm Inc., Fort Lauderdale, FL). The wounds were then covered by a semioclusive Tegaderm dressing, 6 × 7 cm (3M, St. Paul, MN), fixed to the body with Nexaband tissue adhesive (Closure Medical Corp., Raleigh, NC). The mice were housed individually after surgery.

Mice were divided into four groups (*n* = 11 to 12 mice per group). Groups 1 and 2 received i.p. injections of LSKL peptide (30 mg/kg body weight, 350 μ L per injection) and saline (350 μ L per injection), respectively, three times a week, starting from 1 week before surgery until the day of sacrifice. Groups 3 and 4 received s.c. injections at the wound edge with either anti-TGF- β neutralizing antibody (1D11) in sterile saline or nonimmune mouse IgG (50 μ g per injection in a total volume of 40 μ L distributed over three injection sites), three times a week, beginning the day of surgery until sacrifice. In another series of studies, mice received s.c. injections of saline instead of nonimmune IgG. The extent of wound closure was measured using a caliper every 2 or 4 days after the surgery. The average diameter through four dimensions was used to calculate the gross area of the unhealed wound. Animals were sacrificed at days 4, 10, or 22 after wounding, and six to eight wounds per time point in each group were harvested.

Histologic and Morphometric Analysis

Kidneys were fixed by immersion in 4% paraformaldehyde-PBS overnight at 4°C and were processed routinely into paraffin blocks. Duplicate sections from each block were cut 5- μ m thick and stained with H&E or were cut 3- μ m thick and stained with PAS. For histomorphometry, digital images of PAS-stained glomeruli were captured using a Nikon Eclipse E600 microscope (Nikon Instruments, Melville, NY) using the 40× objective and a SPOT Insight camera (SPOT Imaging Solutions, a division of Diagnostic Instruments Inc., Sterling Heights, MI). Ten

Table 1. Characteristics of C57BL/6 and Akita C57BL/6 Mice Treated with Low-Dose (3 mg/kg) Peptide

Treatment	Body weight (g)	Blood glucose (mg/dL)	Systolic blood pressure (mmHg)	24-hour urine volume (mL)
BL/6 Mice				
Before treatment				
Saline (<i>n</i> = 20)	27.8 ± 0.4	96 ± 23	n.t.	n.t.
LSKL (<i>n</i> = 20)	27.1 ± 0.5	85 ± 20	n.t.	n.t.
SLLK (<i>n</i> = 20)	26.4 ± 0.6	80 ± 17	n.t.	n.t.
8 weeks of treatment			n.t.	n.t.
Saline (<i>n</i> = 20)	29.8 ± 0.5	94 ± 25	n.t.	n.t.
LSKL (<i>n</i> = 20)	29.2 ± 0.6	87 ± 18	n.t.	n.t.
SLLK (<i>n</i> = 20)	28.4 ± 0.6	79 ± 18	n.t.	n.t.
15 weeks of treatment				
Saline (<i>n</i> = 20)	29.8 ± 0.5	96 ± 24	108 ± 3	0.8 ± 0.4
LSKL (<i>n</i> = 20)	29.8 ± 0.6	100 ± 14	105 ± 4	0.9 ± 0.4
SLLK (<i>n</i> = 20)	29.0 ± 0.6	95 ± 12	105 ± 4	0.7 ± 0.3
Akita Mice				
Before treatment				
Saline (<i>n</i> = 19)	21.2 ± 1.7	424 ± 52	n.t.	n.t.
LSKL (<i>n</i> = 20)	21.5 ± 1.6	369 ± 96	n.t.	n.t.
SLLK (<i>n</i> = 19)	20.8 ± 2.0	346 ± 71	n.t.	n.t.
8 weeks of treatment				
Saline (<i>n</i> = 19)	24.1 ± 1.4	377 ± 80	n.t.	n.t.
LSKL (<i>n</i> = 20)	24.1 ± 1.5	368 ± 101	n.t.	n.t.
SLLK (<i>n</i> = 19)	24.1 ± 1.8	341 ± 102	n.t.	n.t.
15 weeks of treatment				
Saline (<i>n</i> = 18)	22.2 ± 1.0	397 ± 104	106 ± 5 (<i>n</i> = 18)	3.2 ± 1.4 (<i>n</i> = 18)
LSKL (<i>n</i> = 19)	21.7 ± 1.2	390 ± 71	100 ± 5 (<i>n</i> = 18)	3.6 ± 2.0 (<i>n</i> = 18)
SLLK (<i>n</i> = 17)	22.8 ± 0.9	394 ± 104	103 ± 4 (<i>n</i> = 17)	3.6 ± 2.1 (<i>n</i> = 17)

Data are given as mean ± SD.
n.t., not tested.

Table 2. Characteristics of Akita Mice during High-Dose Peptide Treatment (30 mg/kg Body Weight)

Treatment	Body weight (g)	Blood glucose (mg/dL)	Systolic blood pressure (mmHg)	24-hour urine volume (mL)
Before treatment				
Saline	19.7 ± 1.0 (10)	404 ± 110 (10)	109 ± 6 (10)	1.5 ± 0.5 (10)
LSKL	20.8 ± 1.3 (10)	375 ± 65 (10)	105 ± 12 (10)	2.2 ± 0.9 (10)
SLLK	20.8 ± 1.2 (10)	378 ± 56 (10)	108 ± 10 (10)	1.8 ± 0.5 (10)
8 weeks of treatment				
Saline	23.3 ± 2.2 (10)	332 ± 80 (10)	127 ± 5 (10)	2.4 ± 0.7 (10)
LSKL	23.0 ± 2.3 (9)	373 ± 72 (10)	133 ± 9 (10)	3.0 ± 1.5 (10)
SLLK	23.4 ± 1.4 (10)	437 ± 80 (10)	127 ± 6 (10)	3.2 ± 1.1 (10)
15 weeks of treatment				
Saline	23.5 ± 2.2 (9)	321 ± 114 (9)	96 ± 7 (9)	3.4 ± 0.8 (9)
LSKL	23.0 ± 1.9 (9)	353 ± 117 (9)	94 ± 8 (9)	3.2 ± 1.5 (9)
SLLK	22.9 ± 2.0 (10)	388 ± 137 (10)	96 ± 8 (9)	4.1 ± 1.6 (10)

Data are given as mean ± SD. Values in parentheses are the number of animals tested per group.

glomeruli per mouse were evaluated. Glomeruli were selected that had open capillary lumina, were sectioned through the center of the tuft, and were free of artifacts. Selection bias was minimized by beginning at the point at which the margin of the section was uppermost in the microscope field, moving clockwise to scan the outer cortex, and selecting the first acceptable glomerulus. Remaining glomeruli were selected by continuing around the cortex and selecting approximately equal numbers of glomeruli from the outer, middle, and inner zones of the cortex, avoiding the very large innermost glomeruli. Before analysis, the contrast, brightness, and sharpness of the images were adjusted as necessary to allow the operator to best visualize mesangium and basement membranes. Total and PAS-stained areas of each glomerulus were measured using Image-Pro Plus v6.2 software. PAS-stained areas were measured two ways: in color images using color segmentation and in grayscale images by thresholding. Data were captured using Excel spreadsheets (Microsoft Corp., Redmond, WA), and ratios of PAS-stained area/total glomerular area were calculated for each image.

For analysis of the number of phospho-Smad2-positive nuclei per glomerulus, at least 20 cortical glomeruli

per mouse were evaluated in 2- μ m paraffin-embedded sections at $\times 40$ magnification. Sections from four mice per group were analyzed. Glomerular hilar cross sections with visible afferent and efferent arterioles were captured using a Nikon Eclipse TE2000-U microscope (Nikon Instruments) equipped with a QImaging digital camera (QImaging, Surrey, BC, Canada). Staining for fibronectin was evaluated in two $\times 20$ microscopic fields per mouse that contained cortical through medullary regions. Four to seven animals per condition were analyzed. Fibronectin-stained area and number of phospho-Smad2-positive nuclei were analyzed using MetaMorph version 6.2r4 image analysis software (Universal Imaging Corp., Downingtown, PA). The observer was blinded to the experimental conditions.

Skin wound samples from *db/db* mice were fixed in 4% paraformaldehyde for 72 hours at 4°C. They were embedded in paraffin and sectioned at 5- μ m thickness. The H&E-stained tissues were examined under the Nikon microscope, and digital photographs were captured. The muscle edges of the panniculus carnosus were used as an indicator for the wound edges. Granulation tissue was determined as a zone between the lateral wound edges, superiorly to the s.c. adipose

Figure 1. Urinary albumin excretion and albumin/creatinine ratio. Twenty-four-hour urinary albumin excretion in Akita mice (A and C) and C57BL/6 mice (B) measured using the Albuwell M kit after 15 weeks of low-dose (3 mg/kg) (A) and high-dose (30 mg/kg) (C) peptide treatments. Results are expressed as mean ± SEM (error bars) ($n = 17$ to 19 for low dose; $n = 9$ to 10 for high dose and C57BL/6). * $P = 0.008$ by one-way analysis of variance with Tukey post hoc analysis for saline versus high-dose LSKL. Albumin/creatinine ratios measured using the Creatinine Companion kit in 24-hour urine samples of Akita mice (B and D) and C57BL/6 mice (B) after low-dose (B) and high-dose (D) peptide treatments. Results are expressed as mean ± SEM (error bars) ($n = 17$ to 19 for low dose; $n = 9$ to 10 for high dose and C57BL/6). * $P = 0.003$ for saline versus high-dose LSKL (one-way analysis of variance with Tukey post hoc analysis).

Figure 2. LSKL peptide treatment increases nephrin expression and reduces ED-A fibronectin levels in renal lysates. Nephrlin and ED-A fibronectin protein levels in renal lysates from Akita mice treated with high-dose peptides were examined by immunoblotting and protein levels normalized to GAPDH. Results of densitometric analyses are expressed as mean $\pm$ SD (error bars) ($n = 4$). * $P = 0.023$ LSKL versus saline; ** $P = 0.025$ LSKL versus SLLK (one-way analysis of variance with Tukey analysis). Differences in fibronectin levels were not statistically significant.

tissue and inferiorly to the reepithelialized epidermis (if not at an open wound surface). The granulation tissue area at midline levels across the wounds was quantified in pixels at $\times 40$ magnification in a blinded manner using MetaMorph version 6.2 r4 software. The number of pixels was then divided by the wound length as measured between two wound edges in each section. To quantify the number of phospho-Smad2-positive nuclei or the number of F4/80-positive cells, the number of total nuclei and the number of positive-staining cells were counted by an observer blinded to the experimental conditions in two randomly selected $40\times$ microscopic fields from each wound.

IHC Analysis

Paraformaldehyde (4%)-fixed paraffin sections of kidney and skin wounds were used for IHC staining.

Figure 3. LSKL peptide reduces renal fibronectin staining in Akita mice. Low-magnification ($\times 20$) micrographs of antifibronectin-stained kidney sections that spanned the cortical through the medullary regions were obtained from high-dose-treated Akita mice after 25 weeks of treatment. Two sections per animal were examined [$n = 4$ to 7 animals per group (saline: $n = 6$, LSKL: $n = 7$, and SLLK: $n = 4$)]. The number of pixels positive for fibronectin staining was measured in these sections. Results are expressed as mean $\pm$ SD (error bars) number of pixels per $\mu\text{m}^2 \times 100$. * $P = 0.037$ LSKL versus saline; $P = 0.001$ LSKL versus SLLK (one-way analysis of variance with Tukey analysis).

Briefly, antigen unmasking was performed on deparaffinized slides. For phospho-Smad2 and fibronectin detection, kidney sections were immersed in 10 mmol/L sodium citrate buffer (pH 6.0) in a 99°C water bath for 20 minutes and then were cooled for 20 minutes or, alternatively, skin sections were microwaved for 10 minutes. Endogenous peroxidase activity was quenched with 1% hydrogen peroxide (Sigma-Aldrich Corp.) in PBS. Tissues were blocked with 5% horse serum (Sigma-Aldrich Corp.). Sections were then incubated with the primary antibodies anti-phospho-Smad2 (1:50 for kidney and 1:400 for skin wound) or antifibronectin (1:4000) overnight at 4°C in a humidity box. Primary antibody was either omitted or substituted with preimmune IgG as negative controls. After washing in PBS, appropriate biotinylated secondary antibodies (Vector Laboratories, Burlingame, CA) were applied to the tissue for 1 hour at room temperature, followed by treatment with avidin-biotin complex (Vector Laboratories) for 30 minutes at room temperature at dilutions recommended by the manufacturer. Processed sections were developed with 3,3'-diaminobenzidine hydrochloride (Vector Laboratories) and then were counterstained with hematoxylin. After dehydration, the slides were mounted in VectaMount mounting medium (Vector Laboratories). Wound sections were subjected to antigen retrieval with proteinase K solution (3 minutes, room temperature), treated with 1% hydrogen peroxide, and blocked with 5% normal goat serum before incubation with $0.1 \mu\text{g}/\text{mL}$ goat anti-mouse F4/80 antigen IgG overnight at 4°C . Washed sections were incubated with biotin-labeled goat anti-rat IgG at 1:400 (Vector Laboratories) and were color developed using the avidin-biotin complex/3,3'-diaminobenzidine hydrochloride protocol as described previously herein.

Statistical Analysis

Statistical analyses were performed using SigmaStat 3.1.1 software (SPSS Inc., Chicago, IL) using one-way analysis of variance with Student-Newman-Keuls or Tukey post hoc analysis or the Student's t -test. A $P < 0.05$ was considered statistically significant.

Figure 4. PAS micrographs of representative glomeruli. PAS-stained kidney sections with methylene green counterstain from Akita mice after 15 weeks of i.p. treatment with saline or LSKL or SLLK peptide (3 mg/kg per injection). Original magnification, $\times 40$.

Results

Characteristics of Akita Mice

Akita mice are known to be hyperglycemic by 10 to 11 weeks of age compared with age-matched C57BL/6 mice.⁴⁵ Blood glucose levels were also measured at the start of treatment (13 to 15 weeks old) and after 8 and 15 weeks of treatment, and glucose levels were elevated in Akita mice during the 15 weeks of treatment (age, 25 weeks) (Tables 1 and 2). Body weights of the Akita mice were also significantly reduced compared with those of C57BL/6 mice ($P < 0.001$; Table 1). All the groups showed weight gain during the first 8 weeks of treatment, and the three Akita groups receiving low-dose treatment had a slight decrease in weight at 15 weeks irrespective of treatment. The Akita groups receiving high-dose treatment did not show weight loss (Table 2). Systolic blood pressure was not elevated in Akita mice versus control C57BL/6 mice at 15 weeks of treatment (Table 1). Akita mice receiving high-dose treatment had elevated blood pressure at 8 weeks ($P < 0.001$), which declined at 15 weeks irrespective of treatment (Table 2).

Treatment with LSKL Peptide Reduces Albuminuria

Mice underwent uninephrectomy at 11 to 13 weeks of age and were allowed to recover from surgery for 2 weeks before initiation of treatments. Uninephrectomy has been shown to accelerate the development of renal complications and activate the renin-angiotensin system.⁴⁶ To test the effects of inhibition of TSP1-dependent latent TGF- β activation on the development of renal complications of diabetes, mice were treated by i.p. injections of LSKL peptide in sterile saline three times per week for 15 weeks. Akita/BL6 mice and C57BL/6J mice received treatment with 3 mg/kg peptide (low dose) per injection ($n = 20$ per group). In addition, either control peptide SLLK or saline was administered as a control for LSKL treatment. In a second series of animals, Akita/BL6 mice were treated for 15 weeks with a higher dose of LSKL or SLLK peptide (30 mg/kg/injection, $n = 10$ per group; high dose). During week 15 of treatment, 24-hour urine samples were collected from the mice from each group for analysis of urinary albumin and creatinine levels and TGF- β activity. The treatments had no effect on blood glucose levels or body weight (Tables 1 and 2). Low-

Table 3. Glomerulosclerosis at 15 Weeks of Treatment

Group	No. per group	Glomerular area (pixels)	PAS-positive area (pixels)	PAS-positive/total area (%)
3-mg/kg dose				
C57BL/6 + saline	6	72,514 $\pm$ 6595	16,470 $\pm$ 4231	22.6 $\pm$ 4.4
C57BL/6 + LSKL	6	77,791 $\pm$ 7613	17,888 $\pm$ 4726	23.1 $\pm$ 6.1
C57BL/6 + SLLK	6	67,672 $\pm$ 11,591*	15,242 $\pm$ 3851	22.7 $\pm$ 5.1
Akita + saline	17	87,924 $\pm$ 18,704	24,196 $\pm$ 5485†	27.4 $\pm$ 4.7
Akita + LSKL	19	94,705 $\pm$ 12,122	28,530 $\pm$ 5190‡	30.4 $\pm$ 5.5§
Akita + SLLK	18	93,465 $\pm$ 10,982	25,441 $\pm$ 4725§	27.3 $\pm$ 4.6
30-mg/kg dose				
Akita + saline	9	85,748 $\pm$ 8605	23,998 $\pm$ 5787†	28.4 $\pm$ 7.7
Akita + LSKL	9	93,209 $\pm$ 12,965	24,229 $\pm$ 3556†	26.1 $\pm$ 2.8
Akita + SLLK	9	88,503 $\pm$ 7450	22,607 $\pm$ 3157†	25.4 $\pm$ 2.0

Data are given as mean $\pm$ SD. Analysis of variance with Student-Newman-Keuls post hoc analysis.

* $P < 0.05$ versus low-dose Akita saline, LSKL, SLLK; and high-dose saline, LSKL, SLLK.

† $P < 0.05$ versus C57BL/6 saline and SLLK.

‡ $P < 0.05$ versus all C57BL/6 groups and high-dose SLLK.

§ $P < 0.05$ versus all C57BL/6 groups.

Figure 5. Phospho-Smad2 levels are reduced in renal lysates of Akita mice treated with low- and high-dose LSKL peptide. Akita mice treated with saline or peptides for 15 weeks were evaluated for phospho-Smad2 (high dose), phospho-Smad2/3 (low dose), total Smad2/3, and GAPDH by immunoblotting of renal lysates. Blots were analyzed by densitometric analyses, and the ratio of phospho-Smad2 or phospho-Smad2/3 to total Smad2/3 was normalized to GAPDH. Results are expressed as mean $\pm$ SD (error bars) ($n = 4$). Low dose: * $P = 0.015$ LSKL versus saline; $P = 0.011$ LSKL versus SLLK (one-way analysis of variance with Tukey). High dose: ** $P = 0.001$ LSKL versus SLLK; $P = 0.002$ LSKL versus saline (one-way analysis of variance with Tukey).

dose, but not high-dose, LSKL treatment reduced blood pressure at 15 weeks compared with saline-treated Akita mice ($P = 0.002$). Mice treated with LSKL peptide had reduced 24-hour albumin excretion and reduced albumin/creatinine ratios compared with mice treated with either saline or control peptide (Figure 1). Reductions in urinary albumin levels and albumin/creatinine ratios were statistically significant in mice treated with high-dose LSKL peptide (Figure 1, C and D). In contrast, neither LSKL nor control SLLK peptide affected urine albumin levels and albumin/creatinine ratios in C57BL/6J mice treated with low-dose peptides (Figure 1, A and B). Twenty-four-hour urine volumes in high-dose Akita mice measured during the treatments showed increased urine volumes between the start of treatment and 8 to 15 weeks ($P < 0.05$), with Akita mice having significantly increased urine volumes compared with C57BL/6 control mice ($P < 0.01$), but volumes did not differ between treatment groups with either high- or low-dose peptide (Tables 1 and 2). Serum creatinine levels were also measured to assess renal function. LSKL treatment (30 mg/kg) had no effect on mean $\pm$ SD serum creatinine levels versus saline treatment in Akita mice (0.15 ± 0.5 mg/dL versus 0.19 ± 0.06 mg/dL), although serum creatinine levels were not significantly elevated in either high- or low-dose Akita saline controls compared with in C57BL/6 mice (data not shown).

LSKL Treatment Reduces Renal Injury

Because podocyte function is critical for maintenance of the glomerular filtration barrier and LSKL treatment reduced proteinuria, we asked whether blockade of TSP1-dependent TGF- β activation reduces podocyte dysfunction. Renal lysates were immunoblotted with antibody to the podocyte protein nephrin, a marker of the podocyte slit diaphragm and barrier function (Figure 2).⁴⁷ Akita mice treated with 30 mg/kg LSKL had significantly increased nephrin expression, greater than twofold, compared with renal lysates from either saline controls or SLLK-treated mice, suggesting that LSKL blockade of TGF- β prevents damage to podocyte barrier function.

Fibronectin is a marker of early diabetic nephropathy and a marker of tubulointerstitial injury associated with TSP1 expression.^{48,49} Furthermore, TSP1 increases fibronectin expression by cultured mesangial cells in a TGF- β -dependent manner.⁵⁰ Therefore, the effects of LSKL treatment on renal fibronectin expression were examined by immunostaining of renal sections and by immunoblotting of renal lysates. Immunoblotting of renal lysates from high-dose LSKL-treated mice showed a >66% reduction in fibronectin expression (Figure 2). Fibronectin staining was significantly reduced in renal sections from high-dose LSKL-treated mice compared with either saline- or control SLLK peptide-treated mice (Figure 3). Low-dose LSKL treatment also reduced fibronectin staining

Figure 6. Phospho-Smad2-positive nuclei are reduced in high-dose peptide-treated Akita mice. Sections of Akita mouse kidneys treated for 15 weeks with high-dose peptides (30 mg/kg) were immunostained for phospho-Smad2, and the number of positive nuclei was counted in at least 20 glomeruli per mouse ($n = 4$ per group). Results are expressed as mean $\pm$ SD (error bars). * $P < 0.002$ LSKL versus saline; $P = 0.002$ LSKL versus SLLK (one-way analysis of variance with Tukey post hoc analysis).

Figure 7. Urinary TGF- β activity is reduced in LSKL-treated mice. TGF- β activity in 24-hour urine samples collected after 15 weeks of low-dose (**A**) and high-dose (**B**) peptide treatment was measured using the plasminogen activator inhibitor-1 luciferase promoter reporter assay. Results are expressed as mean $\pm$ SEM (error bars) ($n = 7$ to 8 for high dose; $n = 8$ to 11 for low dose). * $P = 0.007$ LSKL versus saline; ** $P \leq 0.001$ LSKL versus saline (one-way analysis of variance with Tukey post hoc analysis).

and protein in lysates, although this reduction did not reach statistical significance (data not shown).

Mice were also evaluated for glomerular size and glomerulosclerosis as determined by PAS staining (Figure 4 and Table 3). Glomerular area was significantly increased in control SLLK peptide-treated Akita mice (high and low dose) compared with in SLLK-treated C57BL/6 mice. However, neither saline-treated nor LSKL-treated Akita mice showed increases in glomerular area compared with similarly treated C57BL/6 mice. Measurement of PAS-stained area, an indicator of glomerulosclerosis, showed a statistically significant increase in saline-treated Akita mice from the low- and high-dose studies compared with in saline-treated C57BL/6 mice, indicative of increased glomerular fibrosis in this model of diabetic nephropathy. Akita mice treated with either high- or low-dose control (SLLK) peptide also had increased PAS-stained areas compared with nondiabetic C57BL/6 control mice, and PAS staining of SLLK-treated mice did not differ from that of saline-treated Akita mice, suggesting that the control peptide has no effect on glomeruloscle-

rosis. Although treatment with LSKL peptide reduced proteinuria and markers of renal injury in Akita mice, there was no significant reduction in PAS-stained area in either high- or low-dose LSKL-treated Akita mice compared with saline- or SLLK-treated Akita mice (Table 3).

LSKL Peptide Reduces Active TGF- β in Tissues and Urine

Consistent with reduced tubulointerstitial fibronectin and increased nephrin staining, LSKL-treated mice showed a reduction in TGF- β activity in the kidney. Lysates of renal tissues showed significant reductions in phospho-Smad2 levels in LSKL-treated mice compared with in either saline- or control SLLK peptide-treated mice (Figure 5). These reductions were significant for low- and high-dose-treated mice, and the extent of phospho-Smad2 reduction by LSKL peptide was dose dependent. Correspondingly, the number of phospho-Smad2-positive nuclei was also reduced in glomeruli of high-dose LSKL-treated

Figure 8. LSKL i.p. does not affect wound closure rates or granulation tissue formation. **A:** Wounds from diabetic (BKS.Cg-m^{+/+} *Lepr^{db}*/J) animals receiving i.p. injection of LSKL or saline or s.c. injection of either nonimmune mouse IgG or anti-TGF- β IgG were evaluated for wound closure over time. Each point represents the mean $\pm$ SD (error bars) percentage area of the original wound size. Seven to eight wounds per time point were analyzed as described in *Materials and Methods*. One-way analysis of variance using Student-Newman-Keuls analysis. Day 4: $P = 0.025$ anti-TGF- β versus saline; day 10: $P = 0.009$ anti-TGF- β versus LSKL; day 12: $P = 0.013$ anti-TGF- β versus saline, $P = 0.013$ anti-TGF- β versus LSKL, $P = 0.011$ anti-TGF- β versus IgG; day 14: $P = 0.006$ anti-TGF- β versus saline, $P = 0.004$ anti-TGF- β versus LSKL; day 18: $P = 0.038$ anti-TGF- β versus saline, $P = 0.028$ anti-TGF- β versus LSKL, $P = 0.05$ TGF- β versus IgG; and day 22: $P = 0.037$ TGF- β versus saline, $P = 0.027$ TGF- β versus LSKL. **B:** Granulation tissue area was measured in the midsections of diabetic wounds at day 10 after wounding ($n = 7$ to 8 wounds per group). Results are expressed as the mean $\pm$ SD (error bars) granulation tissue area per wound length. * $P = 0.046$ saline versus anti-TGF- β ; $P = 0.027$ LSKL versus anti-TGF- β ; $P = 0.019$ IgG versus anti-TGF- β using the Student's *t*-test.

Figure 9. LSKL treatment does not reduce F4/80 macrophage staining in wounds. Day 10 wound sections were analyzed for macrophage infiltration by staining for F4/80 antigen in mice treated with i.p. injection of SLLK or LSKL peptide or s.c. injection of saline or monoclonal anti-TGF- β neutralizing antibody. Results are the mean $\pm$ SD (error bars) percentage of nuclei that are F4/80 positive per 40 $\times$ field; cells were evaluated in two high-power fields (hpf) per wound in one to two wounds per mouse and in four mice per group ($n = 8$ wounds for SLLK, $n = 8$ wounds for LSKL, $n = 4$ wounds for saline, and $n = 4$ wounds for anti-TGF- β treatments). $P = 0.021$ s.c. anti-TGF- β versus i.p. LSKL (one-way analysis of variance with Tukey analysis).

mice compared with in those of controls (Figure 6). TGF- β activity in the urine of low- and high-dose LSKL-treated mice was also reduced: high-dose LSKL reduced urinary TGF- β levels by $\sim 85\%$, and low-dose LSKL reduced levels to $\sim 50\%$ of those measured in saline-treated Akita mice. Control SLLK peptide at the high dose also reduced active TGF- β by $\sim 25\%$, whereas control peptide at the low dose had no effect (Figure 7).

Systemic Effects on Inflammation and Tumor Incidence at Necropsy

Tissues from Akita mice treated with high-dose peptide were systematically examined at the macroscopic and microscopic levels for tumor incidence and inflammation.

Lungs, liver, aorta, pancreas, spleen, intestine, skin, brain, and reproductive organs were examined, and there were no inflammatory infiltrates in any of the organs examined in any of the treatment groups. Furthermore, no tumors were observed in any of the animals.

LSKL Blocking Peptide Does Not Affect Dermal Wound Healing in Diabetic Mice

To assess whether long-term blockade of TSP1-activated TGF- β by the antagonist peptide interferes with the homeostatic functions of TGF- β in wound healing, full-thickness excisional skin wounds were generated in *db/db* mice. This is a well-established model of impaired wound healing in diabetes, and it has previously been shown that treatment of diabetic wounds with active TGF- β improves wound healing.^{51,52} All the wounds were at a similar cranial-caudal position to avoid the possible cranial-caudal differences in healing. The wounds were covered with the semioclusive dressing until harvest to decrease tissue contraction and better assess reepithelialization.⁴⁴ There were no differences in the rate of wound closure more than 22 days after wounding in mice receiving i.p. injections of either LSKL or saline or s.c. nonimmune IgG (Figure 8A). In contrast, s.c. injection of anti-TGF- β neutralizing antibody into the wounds significantly delayed wound closure between days 10 and 22, suggesting that endogenous TGF- β levels contribute to diabetic wound healing (Figure 8A). Furthermore, i.p. treatment with LSKL peptide had no effect on wound granulation tissue formation at either day 10 or 22 after wounding (Figure 8B and data not shown). In contrast, local injection of neutralizing antibody against TGF- β at the wound site impaired granulation tissue formation (Figure 8B). Macrophage infiltration into the wounds on day 10 was not reduced by i.p. LSKL peptide administration compared with that in either SLLK- or saline-treated mice, although macrophage infiltration was reduced by local injection of anti-TGF- β neutralizing antibody compared with that in animals treated with i.p. injections of LSKL (Figure 9).

Figure 10. LSKL treatment does not reduce phospho-Smad2-positive nuclei in wounds. Wound sections from mice receiving i.p. injection of LSKL or saline or s.c. injection of either nonimmune mouse IgG or anti-TGF- β IgG were harvested 10 days after injury, fixed, and stained for phospho-Smad2. **A:** Representative sections of day 10 wound tissue. Original magnification, $\times 40$. **B:** Central regions of the wounds were evaluated for the number of phospho-Smad2-positive nuclei per 40 $\times$ high-power field (hpf). Positive nuclei were quantified using computer-assisted morphometric software. Two high-power fields from each wound section from four mice per group were randomly selected for analysis ($n = 7$ to 8 wounds). Results are expressed as the mean $\pm$ SD (error bars) number of nuclei that are phospho-Smad2-positive. $P = 0.022$ TGF- β versus LSKL; $P = 0.037$ saline versus TGF- β ; $P = 0.051$ IgG versus TGF- β (one-way analysis of variance with Student-Newman-Keuls analysis).

LSKL Treatment Does Not Impair TGF- β Signaling in Diabetic Skin Wounds

To determine whether i.p. LSKL peptide administration affected local TGF- β signaling in the wound bed, wound sections were examined for nuclear phospho-Smad2 on day 10 after wounding. Ten days after wounding, the number of phospho-Smad2-positive nuclei in granulation tissue was similar in mice treated with i.p. injection of either LSKL or saline (Figure 10). However, the local delivery of pan-specific antibody against TGF- β , but not nonimmune IgG, effectively reduced the number of phospho-Smad2-positive nuclei in granulation tissue (Figure 10). This contrasts with the reduction in phospho-Smad2-positive cells in the kidney in LSKL-treated mice (Figure 6).

Discussion

Previous work from our laboratory and others has implicated TSP1 as a significant regulator of latent TGF- β activation in the diabetic milieu and as important for the development of glomerular and tubular damage in the diabetic kidney and diabetic lesions in other organs.^{7,30,35,36,38,39,53,54} In the present studies, we addressed whether selective targeting of only TGF- β activated by TSP1 would be an effective approach to reduce renal dysfunction in a mouse model of type 1 diabetes. These studies showed that i.p. injection of LSKL peptide to suppress TSP1-dependent TGF- β activation reduced active TGF- β in the kidneys and improved renal function of Akita mice as measured by 24-hour urinary albumin level and the albumin/creatinine ratio. LSKL peptide had a dose-dependent effect on proteinuria, with reductions in albuminuria and the albumin/creatinine ratio in treated Akita mice to levels comparable with those in control C57BL/6 mice. Treatment of Akita mice with high-dose LSKL peptide decreased renal fibronectin levels and increased nephrin levels relative to saline- or control peptide-treated Akita mice. There were no effects of the peptides on blood glucose level, body weight, blood pressure, serum creatinine level, or 24-hour urine volume with LSKL treatment. These data suggest that blockade of TSP1-dependent TGF- β activation is effective at reducing proteinuria and renal injury in diabetic animals. Examination of multiple organs at necropsy showed no increase in inflammation or tumor incidence. Furthermore, systemic administration of LSKL peptide did not impair dermal wound healing in a mouse model of type 2 diabetes. Together, these results suggest that specific blockade of TSP1-dependent TGF- β activation represents a viable therapeutic approach to treating diabetic renal disease that does not affect homeostatic TGF- β function.

TGF- β affects podocyte and tubular function in diabetes.⁵ Although TGF- β is known to cause podocyte apoptosis, it can also cause loss of podocyte function, with decreased expression of slit barrier proteins such as nephrin and epithelial mesenchymal transition of podocytes, both of which are associated with loss of barrier

filtration function.^{8,9} The increase in nephrin expression in LSKL-treated Akita kidneys suggests that one mechanism by which LSKL reduces proteinuria is by preventing TGF- β -mediated podocyte damage. This would be consistent with the observations of Daniel et al,³⁶ who showed that diabetic TSP1 knockout mice have increased staining for the podocyte antigen desmin. LSKL treatment of rats with mesangioproliferative glomerulonephritis also preserved podocyte marker expression as measured by Wilms Tumor suppressor gene staining.⁵⁵

There is also ample evidence that TSP1-regulated latent TGF- β activation is important in tubulointerstitial fibrosis.⁴⁸ TSP1 expression is stimulated by glycated albumin treatment of Madin-Darby canine kidney renal tubular cells *in vitro*, and treatment of cells with anti-TSP1 antibodies reduced cellular hypertrophy and TGF- β activity.³⁸ TSP1 mRNA expression is also increased in aged and obese Zucker rat tubulointerstitium.⁵⁶ In streptozotocin-treated endothelial nitric oxide synthase knockout mice, which develop diabetic nephropathy in addition to hypertension, TSP1 expression is increased and correlates with TGF- β expression and tubulointerstitial injury.⁵³ Furthermore, TSP1 protein expression correlated with increased phospho-Smad2/3 levels in the tubulointerstitium of patients with type 2 diabetes and proteinuria.⁵⁷ Fibronectin is a marker of early fibrosis in diabetic nephropathy.⁴⁹ TSP1 increases fibronectin expression by cultured renal tubular epithelial cells through TGF- β -dependent and independent mechanisms⁵⁸ and also in glucose-treated mesangial cells through a TGF- β -dependent mechanism.⁵⁰ Blockade of CD36 through RNA silencing in cultured renal proximal tubular cells reduced bioactive TGF- β 1 and fibronectin in the presence of pro-fibrogenic concentrations of albumin.⁵⁹ Together, these data suggest that TSP1-mediated activation of latent TGF- β is a key factor in diabetic tubulointerstitial fibrosis and proteinuria. The data showing a reduction in renal fibronectin, especially in the interstitium, by LSKL treatment is supportive of a role for TSP1-regulated TGF- β activation in tubulointerstitial fibrosis in diabetes. This is important because it is well established that tubulointerstitial injury, rather than glomerular injury, is a major determinant of renal functional decline.⁶⁰

Proteinuria precedes loss of renal function, and agents that limit urinary protein excretion preserve renal function.^{61,62} TGF- β is known to alter albumin permeability before up-regulation of ECM genes,⁶³ and TGF- β is increased by podocytes exposed to excess intraglomerular albumin/proteins.⁶⁴ Treatment of mesangial cells with high levels of amino acids increases TSP1 expression and TSP1-dependent TGF- β activity,⁶⁵ and glycated albumin increases TSP1 expressed by cultured renal tubular cells, with increases in TSP1-dependent TGF- β activation.³⁸ These data suggest that TSP1 has a significant effect on proteinuria through regulation of TGF- β activity.

Glomerulosclerosis was increased in Akita mice regardless of treatment compared with in C57BL/6 mice, but total glomerular area was not significantly increased (Table 3). These results differ from those of a study by Gurley et al,⁶⁶ who reported increased glomerular volume and mesangial sclerosis in Akita mice. Despite sig-

nificant improvements in renal function, we observed an increase in glomerular area and in PAS-stained area in LSKL-treated Akita mice but no increase in LSKL-treated C57BL/6 mice. Given the significant proteinuria and the mesangial sclerosis in the Akita model and the reduction in proteinuria with LSKL treatment, we had expected a similar improvement in glomerular morphology. Other direct anti-TGF- β antagonists used in rodent diabetic models, primarily neutralizing antibodies such as 1D11 and CAT-192, reduce proteinuria and/or glomerulosclerosis.¹⁴ In one study, the 1D11 antibody reduced mesangial sclerosis, although proteinuria was not significantly improved.¹² The timing of anti-TGF- β administration also affects outcomes in streptozotocin-treated rats with early treatment (27 to 52 weeks) but not late treatment (52 to 61 weeks), having significant beneficial effects on glomerulosclerosis and proteinuria.⁶¹ The present findings are unusual in that mesangial sclerosis was not affected by LSKL treatment but albuminuria was reduced. Given the known effects of TSP1 and LSKL peptide on blockade of glucose-stimulated TGF- β activity and matrix production by cultured mesangial cells and the reduced mesangial sclerosis in diabetic TSP1 knockout mice,^{33,36,64} it is possible that mesangial accessibility of LSKL peptide administered i.p. was insufficient to attenuate glomerulosclerosis, although the effects of LSKL on nephrin expression and glomerular phospho-Smad levels suggest that the peptide is accessible. The degree of glomerulosclerosis (percentage of PAS-positive area) in Akita mice at the start of treatment (2 weeks after uninephrectomy) did not differ from that in C57BL/6 mice (data not shown), suggesting that the initial degree of established fibrosis is not likely to be a significant factor in these studies. However, similar findings were reported in older Dahl salt-sensitive hypertensive rats with established glomerular disease: anti-TGF- β antibody (1D11) blocked proteinuria and reduced renal TGF- β activity but had no effect on glomerulosclerosis or hypertrophy.⁶⁷ It was suggested that the reduction in proteinuria without reduced glomerulosclerosis could be due to blockade of the effects of TGF- β on glomerular permeability but an inability of the antibody to reverse established fibrosis.^{63,67}

To address the effect of long-term LSKL therapy on other TGF- β -mediated functions in diabetes, we studied dermal wound healing in a well-established model of diabetic wound healing in type 2 diabetic mice.⁴⁴ TGF- β signaling is known to be impaired in diabetic skin wounds, resulting in reduced macrophage chemoattraction, angiogenesis, and extracellular matrix deposition with accelerated reepithelialization.^{68,69} Treatment of diabetic wounds with active TGF- β normalizes wound healing.^{51,52,70} The role of TSP1-dependent TGF- β activation in diabetic wound healing is not known. There are conflicting reports regarding the role of TSP1 in mediating latent TGF- β activation by skin fibroblasts and in wounds: one study shows that TSP1 and TGF- β are not increased by high glucose treatment of skin fibroblasts, but another shows that TSP1-soaked collagen sponges implanted s.c. had increased TGF- β activity and fibroblast gel contraction.^{71,72} TSP1 knockout mice have impaired wound healing with decreased TGF- β in wounds, delayed macrophage infiltration, prolonged angiogenesis, persistent gran-

ulation tissue, and decreased collagen content.^{73,74} At least part of this phenotype can be attributed to decreased TGF- β activity because the phenotype is rescued by topical application of the TSP1 TGF- β activating peptide KRFG.⁷⁴ In the present studies, i.p. administration of LSKL peptide did not affect excisional wound healing or macrophage infiltration. Correspondingly, TGF- β activity in the wound bed was not affected by systemic LSKL peptide administration, although local injection of anti-TGF- β neutralizing antibody did reduce wound closure, macrophage infiltration, and Smad phosphorylation. These data suggest that local dermal concentrations of LSKL peptide administered by i.p. injection are not sufficient to affect local dermal latent TGF- β activation or, alternatively, that TSP1 regulation of latent TGF- β activation does not play a significant role in the control of TGF- β activation in diabetic dermal wound healing. Nonetheless, these studies indicate that systemic LSKL treatment would not further impair wound healing in diabetic individuals.

One of the major considerations for therapeutic targeting of TGF- β in chronic diseases such as diabetes is to avoid disruption of homeostatic TGF- β signaling. Examination of mice at necropsy after 15 weeks of LSKL treatment did not show any inflammatory infiltration or tumors in multiple organs. This is important because TSP1 has an apparent role in regulating TGF- β activation that leads to the development of Th17 effector T cells.⁷⁵ TSP1 null mice have reduced Th17 T cells and IL-17 production and attenuated experimental autoimmune encephalitis due to reduced TGF- β activation.⁷⁵ Conversely, TGF- β can regulate immunosuppressive regulatory T cells, and TSP1 has been implicated in regulating TGF- β activation by regulatory T cells.⁷⁶ It remains to be determined whether systemic blockade of TSP1-dependent latent TGF- β activation alters immune cell profiles in diabetic animals.

These findings support the hypothesis that selective targeting of diabetes-induced excess TGF- β through blockade of TSP1-dependent TGF- β activation is an effective approach with minimal adverse effects compared with more global approaches to abrogating TGF- β signaling. Moreover, LSKL treatment did not have any demonstrable effects on tissue homeostasis in wild-type C57BL/6J (data not shown), similar to our observations in normal rats receiving long-term LSKL peptide treatment.³⁹

In conclusion, these studies establish that blockade of TSP1-mediated TGF- β activation is an effective therapeutic target for treatment of the renal complications of diabetes. These studies suggest that blockade of TSP1-mediated TGF- β activation is a selective approach to controlling excessive TGF- β levels in diabetes and lacks the adverse effects associated with global inhibition of TGF- β signaling.

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