Thrombospondin-2 therapy ameliorates experimental glomerulonephritis via inhibition of cell proliferation, inflammation, and TGF-β activation

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Thrombospondin-2 therapy ameliorates experimental glomerulonephritis via inhibition of cell proliferation, inflammation, and TGF-β activation. Am J Physiol Renal Physiol 297: F1299–F1309, 2009. First published September 2, 2009; doi:10.1152/ajprenal.00254.2009.—We recently identified thrombospondin-2 (TSP-2) as an endogenous regulator of matrix remodelling and inflammation in experimental kidney disease by studying TSP-2-deficient mice. In this study, we asked whether systemic TSP-2 overexpression via thigh muscle transfection is able to ameliorate the time course of the anti-Thy1 glomerulonephritis model. After induction of anti-Thy1 nephritis, rats were transfected either with an overexpression plasmid for TSP-2 or lacZ as a control. Biopsies, urine, and blood samples were taken on days 1, 3, and 6 after disease induction. Muscular overexpression of TSP-2 reduced glomerular transforming growth factor (TGF)-β activation and glomerular extracellular matrix formation as determined by collagen IV and fibronectin. In addition, activation of mesangial cells to the myofibroblast-like phenotype was also significantly decreased in TSP-2-overexpressing animals. TSP-2 overexpression inhibited both glomerular endothelial and mesangial cell proliferation, resulting in a reduced glomerular cell number and glomerular tuft area. The inflammatory response, as monitored by T cells and antigen-presenting cells, was reduced significantly by TSP-2 overexpression, but influx of macrophages was unchanged. These data demonstrate TSP-2 as a potential therapeutic agent to inhibit the glomerular proliferative and inflammatory response as well as TGF-β activation and extracellular matrix accumulation in experimental mesangial proliferative glomerulonephritis.

METHODS

Animal model. The animal studies were performed in accordance with the internal animal review board (Regierung von Mittelfranken: 621–2531.31–17/05) and were approved by the national animal review board (Regierung von Mittelfranken: 54–2532–22/01). The animals were housed in a room maintained at 22 ± 2°C, exposed to a 12:12-h dark-light cycle, and fed standard mouse chow (Altromin 1324; Spezialfutterwerke, Lage, Germany) by a single injection of 1 mg/kg of the mouse monoclonal anti-Thy1 antibody OX-7 (European Collection of Animal Cell Cultures, Salisbury, UK). All rats were randomly allocated to two different groups with anti-Thy1 nephritis receiving either prevention gene therapy with TSP-2 (n = 10) or lacZ as control (n = 10). Renal survival biopsies were taken on days 1 and 3, and rats were killed on day 6. In addition, 14 male anti-Thy1 nephritic Sprague Dawley rats were used in a second experiment using seven rats per TSP-2 and lacZ control group for investigation of glomerular isolates. Glomeruli were isolated by a standard sieving procedure.
For gene therapy, we used the pCAGGS-lacZ vector (kindly provided by M. Streit, Department of Surgery, Massachusetts General Hospital and Harvard Medical School, Boston, MA). Briefly, mTSP-2 was cut from a pCDNA3.1 derivative (kindly provided by M. Streit, Department of Surgery, Massachusetts General Hospital and Harvard Medical School, Boston, MA). After injection of plasmid DNA (5 min), muscles were transfected by electroporation with six 100-volt pulses of 50-ms duration (each with 950-ms intervals) applied by oval-shaped electrodes using a square wave electroporator (BTX, ECM 830; Genetherapeutics, Boston, MA). To determine general extracellular matrix formation, sections were also stained with PAS and semiquantitatively scored from zero to four as follows: score 0 = glomerulus without any pink staining, score 1 = glomerulus with little pink staining, score 2 = glomerulus with moderate pink staining, and score 3 = glomerulus almost completely filled with pink staining. Glomerular cell number was determined by counting hematoxylin-stained nuclei per glomerular cross section 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). All immunohistological evaluations were performed in a blinded fashion by two independent observers.

The following antibodies were used in this study: a murine IgM monoclonal antibody (mAb) against the proliferating cell nuclear antigen (PCNA) (PC10; DAKO, Glostrup, Denmark); ED-1, a murine IgG1 mAb to a cytoplasmic antigen present in monocytes, macrophages, and dendritic cells [Serotec, Oxford, UK (20)]; CD3, a rabbit IgG mAb specific for T cells (Labvision, Fremont, CA); CD8a, a murine IgG1 mAb specific for cytotoxic T cells (BD biosciences, Heidelberg, Germany); CD45R, a murine IgG1 mAb specific for B cells (BD biosciences); MHC class II, a murine IgG1 mAb specific for antigen-presenting cells (Serotec); OX-7, a murine IgG1 mAb; monoclonal antibody raised against the NH2-terminal sequence of mouse TSP-2 (kindly provided by P. Bornstein (10)); and TSP-1, a murine IgG1 mAb, clone A6.1 specific for TSP-1 (Labvision, Fremont, CA). The TGF-β system was studied using antibodies to TGF-β1 [rabbit anti-human TGF-β1; Santa Cruz Biotechnology, Santa Cruz, CA (12)]; TGF-β2 [rabbit anti-human TGF-β2; Santa Cruz (12)]; active TGF-β1 [chicken anti-human active TGF-β1 (R&D systems) (12, 13)]; phospho-Smad2/3 [rabbit anti-human Smad2 peptide phosphorylated at Ser137/145; Santa Cruz (12)] or PAI-1, a rabbit polyclonal to human plasminogen activator inhibitor-1 (Santa Cruz); and α-smooth muscle actin (1A4), a murine IgG2a mAb to human α-smooth muscle actin detecting activated MC from mouse, rat, and human [DAKO (26)].

Negative controls for immunostaining included either deletion or substitution of the primary antibody with equivalent concentrations of an irrelevant murine mAb or preimmune rabbit IgG. 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), and color development with diaminobenzidine without nickel chloride and histogreen (Linaris, Wertheim-Bettingen, Germany) for nuclear staining.

Glomerular expression of matrix molecules such as collagen IV and fibronectin was quantified by computerized measurement of the positive-stained glomerular area using the Metavue Imaging System (Visitron Systems, Puchheim, Germany) and with equivalent results by a semiquantitative scoring system (data not shown) as described below. Total TGF-β1, total TGF-β2, active TGF-β, collagen I, α-smooth muscle actin, TSP-1 and PAI-1 were graded semiquantitatively (18) and reflected changes in the area and intensity of mesangial staining: 0 = very weak or absent staining; 1+, weak staining with

Fig. 1. The experimental design of the study is shown.
25% of the glomerular tuft showing focally increased staining; 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. Mesangiolysis was assessed by grading the loss of staining for the MC marker OX-7 semiquantitatively as described above. In addition, the average number of CD3, CD8, myosin heavy chain (MHC) class II, CD45R, ED-1, or PCNA positive cells per glomerular cross section was determined. Phosphorylated Smad2/3 was shown as a percentage of positive nuclei per glomerular cross section counting both positive- and negative-stained nuclei.

**Immunohistochemical double staining.** To determine the number of proliferating mesangial or endothelial cells, double immunostaining for PCNA, a marker of cell proliferation, and for OX-7, a MC specific marker or JG-12, an endothelial specific marker, were performed as described previously (18). The number of proliferating mesangial and endothelial cells was evaluated by counting the number of cells that stained for both PCNA (green) and OX-7 (brown) or JG-12 (brown) cells, respectively, and was expressed as the mean ± SD per glomerular cross section.

**Cell culture.** Rat MC were cultured from isolated glomeruli using a standard sieving procedure (38) and characterized by positive staining for α-smooth muscle actin and α2-integrin and the lack of the epithelial marker synaptopodin, endothelial marker RECA-1, and the macrophage/monocyte marker CD68. MC were grown in DMEM containing 10% FCS, insulin (5 μg/ml), and penicillin/streptomycin at 37°C and 5% CO2.

**Proliferation assay.** Proliferation of MCs treated with 0.0001–10 μg human TSP-2 (identity of amino acid sequence from predicted rat sequence to human TSP-2 was 88%) (R&D systems) was monitored by Bromodeoxyuridine (BrdU) uptake during S Phase using a cell proliferation ELISA (colorimetric) purchased from Roche Diagnostics (Mannheim, Germany) following the manufacturer’s instructions. Two thousand rat MCs were seeded per well of a microtiter plate and starved for 3 days in DMEM culture medium lacking FCS. Pulsing with BrdU was done for 4 h 1 day after stimulation with 5% FCS. Mean proliferative cell activity from three independent experiments was shown by normalizing the positive control to 100%.

**Apoptosis assay.** Apoptotic cells were detected by TUNEL assay, as described previously (10).

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Fig. 2. Endogenous thrombospondin-2 (TSP-2) expression in healthy and nephritic glomeruli. Endogenous renal TSP-2 expression was evaluated in biopsies from healthy rats treated with the overexpression plasmid for lacZ control (A) or TSP-2 (B) using immunohistochemistry. TSP-2 expression was localized in the brush border of proximal tubular cells (A and B; arrow indicates TSP-2-positive staining). After induction of anti-Thy1 nephritis (6 days), TSP-2 was detected in glomeruli, showing a mesangial staining pattern irrespective of treatment with lacZ (C) or TSP-2 (D) overexpressing plasmid. <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. Mesangiolysis was accessed by grading the loss of staining for the MC marker OX-7 semiquantitatively as described above. In addition, the average number of CD3, CD8, myosin heavy chain (MHC) class II, CD45R, ED-1, or PCNA positive cells per glomerular cross section was determined. Phosphorylated Smad2/3 was shown as a percentage of positive nuclei per glomerular cross section counting both positive- and negative-stained nuclei.

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Fig. 3. Overexpression of TSP-2 resulted in TSP-2-positive muscle cells, enhanced TSP-2 plasma levels, and ameliorated kidney function. Muscles transfected with TSP-2 overexpression plasmid resulted in TSP-2 expression on day 6, as demonstrated by immunohistochemistry (A), whereas muscles transfected with the lacZ control plasmid did not express TSP-2 (B). Only in muscles transfected with β-galactosidase as control lacZ assay shows expression of β-galactosidase within the muscle (D), whereas muscles treated with the TSP-2 overexpression plasmid lacked β-galactosidase activity (C). Gene therapy with TSP-2 resulted in significantly enhanced TSP-2 plasma levels (filled bars), as demonstrated by direct enzyme-linked immunosorbent assay (ELISA; E) without effects on mesangiolysis on day 1 (F). Kidney function was evaluated by measurement of creatinine clearance (G) and 24-h proteinuria (H) on days 3 and 6. *Statistically significant difference between groups treated with gene therapy (P < 0.02).
β-Galactosidase assay. Frozen sections from transfected muscles of 10 μm thickness were fixed for 10 min in 0.05% glutaraldehyde. After washing in PBS, β-galactosidase was detected after incubation for 5 h at 37°C using substrate buffer containing 1 mg/ml x-Gal in 20 mM K₃Fe(CN)₆, 20 mM K₄Fe(CN)₆, and 1.5 mM MgSO₄, pH 8.

Zymography. For zymography, proteins from glomeruli were isolated on days 3 and 6 after disease induction and homogenized in 1% (wt/vol) SDS containing 100 mM Tris, pH 6.8. After sedimentation of non-soluble particles by centrifugation at 14,000 (wt/vol) SDS containing 100 mM Tris, pH 6.8. After sedimentation of non-soluble particles by centrifugation at 14,000 g, aliquots of 20 mg protein were separated at 4°C by 10% SDS-PAGE with 0.06% (wt/vol) gelatin in the resolving gel. SDS was removed by agitation of gels for 120 min in 1% (wt/vol) Triton X-100 in 100 mM Tris, pH 7.5, followed by three subsequent washings for 15 min in 100 mM Tris, pH 7.5. After incubation in the same buffer for 18 h at 37°C, gels were stained with 0.1% (wt/vol) Coomassie brilliant blue R250 in methanol-acetic acid-H₂O (40:10:50) and agitated in the same solution until the stacking gel was destained.

Direct TSP-2 ELISA. Recombinant mouse TSP-2 (kindly provided by P. Bornstein, University of Washington, Seattle, WA) was used for a standard curve using concentrations between 5 and 400 ng/ml. Mouse TSP-2 and an antibody raised against mouse TSP-2 were used to determine total (rat and mouse) TSP-2 serum levels, since there is no rat specific anti-TSP-2 antibody available and identity of amino acid sequence from the predicted rat sequence to mouse TSP-2 is 97%. Recognition of rat TSP-2 by this antibody was proven by Western blot analysis. Standards and plasma samples (1:100) were diluted in PBS and coated on microtiter plates (Nunc immunoplate; Nunc, Roskilde, Denmark) overnight at 4°C, followed by blocking with 1% BSA (Serva, Heidelberg, Germany) and 1% milk powder (Applichem, Darmstadt, Germany) diluted in PBS for 1.5 h. After three rinses with 200 μl PBS/well, anti-mouse TSP-2 antibody (kindly provided by P. Bornstein) diluted 1:500 in PBS was incubated for 1 h. After removal of nonbound primary antibody, plates were incubated with horseradish peroxidase-labeled donkey anti-rabbit antibody (GE Healthcare, Little Chalfont Buckinghamshire, UK) diluted 1:200 in PBS. Recognition of bound primary antibody was detected after 1.5 h by addition of peroxidase substrate consisting of 0.4 mg/ml o-phenylenediamine and 0.06% H₂O₂ (both Merck, Darmstadt, Germany) in 50 mM phosphate buffer, pH 5. The enzyme reaction was stopped by adding 0.5 M H₂SO₄, and the optical density was measured at 492 nm in a microplate reader (Tecan sunrise; Tecan, Salzburg, Austria).

Western blot analysis. The proteins were extracted from isolated glomeruli using 50 mM Tris, 1% (vol/vol) Nonidet P-40, 0.25% (vol/vol) sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM Na₂VO₄, 1 mM NaF, and proteinase inhibitor cocktail (Complete; Boehringer, Mannheim, Germany) as extraction buffer. For a better solubility, extracts were sonified for 30 s at 50% power and 50% duty cycle using a Sonopuls HD70 (Bandelin, Berlin, Germany). Twenty micrograms of protein (20 μg) were resolved in SDS-PAGE using gels containing 8% acrylamide for analyzing collagen IV (Southern Bio-technology Associates, Birmingham, AL) and fibronectin (Labvision, Fremont, CA). Proteins were transferred semi-dry to a nitrocellulose membrane within 1 h at 1.5 mA/cm². β-Actin (Abcam, Cambridge, UK) was detected simultaneously as a loading control. Secondary peroxidase-conjugated antibodies were all purchased from Amersham Biosciences (Buckinghamshire, UK). For evaluation of densitometry, AIDA 4.1 software (Raytest Isotopenmessgeräte, Straubenhardt, Germany) was used. Each experiment for Western blot analysis was done in triplicate.

Quantification of glomerular EDA-fibronectin and collagen I by real-time PCR. The isolation of glomeruli and the quantification of glomerular target proteins by real-time PCR were performed as described (38).

The following rat-specific PCR primers were used in this study: EDA-fibronectin (38), forward: 5’-ACATCGTGTAATGCGAATTTTT-3, reverse: 5’-TCGACACGGAAGCAGAAA-3, and probe: 5’-TTGGTCACAGTCAGTGGAACCAATTTG-3; collagen IV α1-chain, forward: 5’-AACGAAAAAGGACACGAGGA-3, reverse: 5’-GCCAGGAATTACCGAAGAGT-3; collagen I α1-chain, forward: 5’-ATGTTCAGCTTGTGGACCTC-3, reverse: 5’-GCAGCTGATCTTGGGATGT; 18S (22), forward: 5’-TGATTAAGTCTC-3 and reverse: 5’-CGATCCAGGGCCTACTA-3.

Miscellaneous measurements. Urinary protein was measured using the Bio-Rad Protein Assay (München, Germany) and BSA (Sigma, Deisenhofen, Germany) as a standard. Creatinine in serum or urine was measured using an autoanalyzer (Beckman Instruments, München, Germany).

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

Endogenous TSP-2 is expressed in nephritic glomeruli on day 6 and was not altered by TSP-2 gene therapy. In healthy rat kidneys, TSP-2 staining was absent within the glomeruli irrespective of whether muscles were treated with TSP-2- or lacZ-overexpressing plasmid (Fig. 2, A and B). Within the tubulointerstitial compartment, TSP-2 staining was observed in the brush border of some proximal tubules (Fig. 2A). Furthermore, TSP-2 overexpression changed neither the renal nor the vascular phenotype of kidneys from healthy rats (data not shown). After disease induction (3 days), glomerular TSP-2 expression was still below detection level (data not shown), but, on day 6, TSP-2 was expressed at low levels within nephritic glomeruli (Fig. 2, C and D). This mesangial staining pattern was similar in both treatment groups, indicating that endogenous TSP-2 expression was not influenced by gene therapy (Fig. 2, C and D).

Overexpression of TSP-2 resulted in TSP-2 production and secretion by muscle cells, causing enhanced TSP-2 plasma levels. Successful transfection of TSP-2-expressing plasmids in the thigh by electroporation could be demonstrated via both immunohistochemical detection of the product in the muscle as well as the detection of increased systemic levels of TSP-2. First, TSP-2 or β-galactosidase was detected within the muscle cells using immunohistological staining or β-galactosidase assay. TSP-2 was exclusively detected in thigh muscles transfected with TSP-2 overexpression plasmid (Fig. 3A) and not in muscles transfected with the lacZ-expressing plasmid (Fig. 3B). In addition, β-galactosidase activity was restricted to muscle cells transfected with the lacZ-plasmid (Fig. 3, C and D).
Second, on day 6 after transfection, TSP-2 plasma levels were significantly enhanced and nearly doubled in rats treated with the TSP-overexpressing plasmid compared with animals treated with control plasmid (Fig. 3E).

Overexpression of TSP-2 did not affect mesangiolysis but ameliorated kidney function. Induction of anti-Thy1 nephritis was not affected by TSP-2 gene therapy, since mesangiolysis was similar in both groups, as monitored by loss of the rat

![Image of healthy controls, gene therapy with TSP-2-overexpressing vector, and gene therapy with lacZ-overexpressing control vector]

![Graphs showing glomerular PAS positivity, glomerular Collagen IV, glomerular fibronectin, collagen IV, and fibronectin mRNA levels]
mesangial marker Thy1 (Fig. 3F). In contrast, TSP-2 gene therapy in nephritic rats significantly ameliorated kidney function, as measured by creatinine clearance (Fig. 3G). In addition, 24 h proteinuria, a hallmark of kidney disease severity, was significantly reduced on days 3 and 6 compared with control animals (Fig. 3H).

TSP-2 overexpression decreased glomerular extracellular matrix formation in rats with anti-Thy1 disease. Amelioration of kidney function was accompanied by reduced glomerular matrix accumulation as evaluated by PAS staining, immunohistochemistry, Western blot analysis, and real-time PCR. In rats treated with the TSP-2-overexpressing plasmid, glomerular PAS positivity was significantly reduced by 52 ± 27% on day 6 after model induction (P < 0.006; Fig. 4C) as shown by representative pictures for nephritic glomeruli from rats treated with TSP-2 (Fig. 4B) and lacZ gene therapy (Fig. 4A). Consistent with this result, specific glomerular matrix molecules were significantly reduced in TSP-2-overexpressing animals on day 6 compared with LacZ-expressing controls (Fig. 4). Accumulation of glomerular collagen IV and fibronectin was reduced in nephritic rats treated with the TSP-2-expressing vector on day 6 after model induction (Fig. 4, E and H) compared with rats treated with the control vector (Fig. 4, D and G) as shown in representative images as well as by computer-assisted quantitation of immunostaining (Fig. 4, F and I). Western blot analysis and real-time PCR using extracts from isolated glomeruli confirmed collagen IV (Fig. 4, J–L), collagen I (Fig. 4L), and fibronectin (Fig. 4, M–O) reduction in rats treated with TSP-2 gene therapy.

TSP-2 overexpression inhibited MC activation toward a myofibroblast-like phenotype. In the anti-Thy1 model, repopulating MCs are in an activated state expressing transiently myofibroblast markers such as α-smooth muscle actin and collagen I. After induction of anti-Thy1 nephritis (6 days), α-smooth muscle actin is homogenously expressed within the regenerating mesangium (Fig. 5A). In contrast, nephritic glomeruli of TSP-2 gene therapy-treated rats showed only focal α-smooth muscle actin expression at the same time point (Fig. 5B). Glomerular α-smooth muscle actin was reduced by >50% on days 3 and 6 (by semiquantitative scoring) in rats treated with the TSP-2-overexpressing plasmid compared with animals treated with the lacZ control plasmid (Fig. 5C). Similar results were found for the expression of glomerular collagen I, which was 3.5 times higher on day 6 in the control vector-overexpressing rats compared with TSP-2-treated animals (Fig. 5, D–F). MMP-2 is shown to be an inducer for the conversion of cells to the myofibroblast phenotype (8). Because TSP-2 has been demonstrated as an endogenous regulator of MMP-2, we also investigated MMP-2 activity using zymography. Gene therapy with the TSP-2 construct significantly reduced MMP-2 activity in glomerular extracts from anti-Thy1 nephritic rats on day 3 (Fig. 5, G and H) but was similar on day 6 (Fig. 5, I and J).

![Images and graphs](http://ajprenal.physiology.org/)

Fig. 5. TSP-2 overexpression reduced mesangial cell activation also by changing glomerular matrix metalloproteinase (MMP)-2 activity. Representative images for immunostaining of α-smooth muscle actin showing strong glomerular expression in rats treated with lacZ plasmid (A, dark gray staining) in contrast to low expression within the glomeruli from TSP-2 plasmid-treated animals (B). Semiquantitative evaluation of α-smooth muscle actin staining revealed significantly reduced expression in TSP-2 plasmid-treated animals compared with lacZ-control animals. This was also shown for collagen I, as assessed by immunohistochemistry (D). Representative images for immunostaining of collagen I showing strong glomerular expression in rats treated with lacZ-plasmid (E, dark gray staining) in contrast to low expression within the glomeruli from TSP-2 plasmid-treated animals (F). MMP-2 activity was monitored by zymography followed by densitometric evaluation on days 3 (G and H) and 6 (I and J). *Statistically significant differences between groups treated with gene therapy (P < 0.04).
TSP-2 gene therapy reduced TGF-β activation while not affecting total TGF-β expression. If TSP-2 is a competitive inhibitor for TSP-1 in the anti-Thy1 model, TGF-β activity in glomeruli from TSP-2-overexpressing plasmid-treated nephritic animals has to be reduced. TSP-1 expression was clearly upregulated during anti-Thy1 nephritis and not significantly different in both treated groups (Fig. 6A). TGF-β activity was monitored using one direct and two different indirect methods. Immunohistochemistry for active TGF-β was conducted by counting the percentage of phospho-Smad2/3-positive cells per glomerular cross section (Fig. 6C) as well as the increased expression of PAI-1 (Fig. 6G, \( P < 0.002 \)) were more than halved on both investigated time points by TSP-2 gene therapy, indicating reduced TGF-β activity. In contrast, total TGF-β1 and TGF-β2 were not affected by TSP-2 gene therapy as assessed by immunostaining (Fig. 6, M and N).

TSP-2 overexpression reduced glomerular proliferation in rats with anti-Thy1 disease. MC proliferation as a hallmark of anti-Thy1 nephritis was also examined in regard to potential effects of TSP-2 gene therapy. Glomerular cell number was similar on day 3 but significantly lower on day 6 in animals treated with the TSP-2-overexpressing plasmid compared with animals treated with the lacZ plasmid (Fig. 7A). Glomerular proliferation, as assessed by staining for the proliferation marker PCNA, was significantly lower on days 3 and 6 in the TSP-2 gene therapy group (Fig. 7B). Double staining for the MC marker OX-7 and the proliferation marker PCNA (Fig. 7D) confirmed significantly reduced numbers of proliferating MC on day 6 (27 ± 19% reduction) at the time of its peak proliferation (Fig. 7C). In contrast, TSP-2-overexpressing nephritic rats exhibited a 50% reduction of the peak glomerular

**Fig. 6.** TSP-2 gene therapy reduced transforming growth factor (TGF)-β activation while not affecting total TGF-β expression. Immunostaining of biopsies was used for semiquantitative evaluation of glomerular expression of TSP-1, active TGF-β, plasminogen activator inhibitor (PAI)-1, total TGF-β1, and total TGF-β2 (A, B, and H–J). TSP-1 expression was similar in anti-Thy1 nephritic animals treated by gene therapy (A and D, dark gray cytosolic TSP-1-positive staining on day 6 showing a glomerulus from a lacZ-treated rat). TGF-β activation was monitored by evaluation of active TGF-β (B), showing gray cytosolic staining in biopsies from lacZ-treated animals (E). In contrast, staining for active TGF-β was much less intensive in rats treated with TSP-2 (F). Evaluation of the phosphorylated (P) TGF-β signaling molecule Smad2/3 was conducted by counting the percentage of P-Smad2/3-positive cells per glomerular cross section (C and G, black nuclear phospho-Smad2/3-positive staining). In addition, the TGF-β downstream target PAI-1 (H) was highly expressed on day 6 in glomeruli from lacZ-treated rats (K, dark gray cytosolic PAI-1-positive staining) and showed only low expression in glomeruli from rats treated with TSP-2 (L). Total TGF-β1 (I and M, gray cytosolic TGF-β1-positive staining in a lacZ-treated rat) and total TGF-β2 (J and N, gray cytosolic TGF-β2-positive staining in a lacZ-treated rat) were similar in both nephritic groups. *Statistically significant differences between groups treated with gene therapy (\( P < 0.002 \)).

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endothelial cell proliferation (double staining for the endothelial marker JG-12 and PCNA) on day 3 (Fig. 7, E and F). These results indicate that TSP-2 affects both mesangial and endothelial cell proliferation during anti-Thy1 nephritis.

To investigate TSP-2 effects on glomerular apoptosis, we performed the TUNEL assay. Glomerular apoptosis was neither changed on day 3 nor on day 6 in either group (Fig. 7, G and H). Suggesting an direct antiproliferative effect of TSP-2 for MC as similarly demonstrated for glomerular endothelial cells (10), incubation of isolated rat MCs resulted in significant reduction of the proliferative response (Fig. 7I). Inhibition of MC proliferation started at concentrations of 0.1 ng/ml TSP-2 and reached a maximum inhibitory effect of 47.6 ± 2.3% when 10 ng/ml were used (Fig. 7I). In addition, the reduced proliferative response by TSP-2 gene therapy without affecting apoptosis resulted in a significantly reduced glomerular tuft area on days 3 and 6 after disease induction (Fig. 7J).

Effects of TSP-2 overexpression on kidney inflammation in experimental glomerulonephritis. Because TSP-2 may regulate renal inflammation, we also determined the number of infiltrating T cells, B cells, and monocytes/macrophages within the glomeruli during anti-Thy1 nephritis (Fig. 8). Highest glomerular infiltration with inflammatory cells was found on day 3 after disease induction (Fig. 8, A, C, E, and G). At this time point, CD3, CD8-positive T cells, and MHCII positive cells were significantly reduced by >50% using TSP-2 gene therapy (Fig. 8, A, C, and E). On day 6 after model induction, infiltration with T cells and MHCII positive cells was lower compared with day 3 but still significantly reduced by TSP-2 therapy (Fig. 8, A, C, and E). In contrast, glomerular macrophages and B cells showed only a tendency to reduced levels after treatment with TSP-2 (Fig. 8, G and I).

**DISCUSSION**

Mesangial proliferative glomerulonephritis is the most common renal disease in the Western world, leading to fibrosis and end-stage renal disease (23). In this study, we could show that treatment with TSP-2 by gene therapy was able to ameliorate experimental glomerulonephritis by several different mechanisms, such as inhibition of matrix accumulation, cellular proliferation, and inflammation.

As the first major mechanism, we could demonstrate that TSP-2 can affect TGF-β activation, the central cytokine mediating renal fibrosis as indicated by various studies in experimental glomerular disease models as well as human biopsy studies (6, 37). The fact that the activation process of the TGF-β procytokine complex required for its action can be...
mediated in different ways dependent on its environment (32) could potentially be used for a more specific antagonistic and therapeutic approach (14). Our group identified TSP-1 as the major endogenous activator of TGF-β in experimental mesangial proliferative glomerulonephritis (12, 13) and in diabetic nephropathy (11). The link of TSP-1 and TGF-β in several experimental kidney disease models (1, 12, 13, 17, 19, 25) as well as the de novo upregulation of TSP-1 in human kidney disease (16, 29) suggest TSP-1 as an excellent target for the treatment of renal fibrosis specifically caused by TSP-1-mediated TGF-β activation.

We believe that TSP-2 overexpression as a therapeutic principle may be advantageous compared with a global TGF-β or TSP-1 blocking therapy, since neither alternate TGF-β activation mechanisms nor other functions of TSP-1 (for example, as an angiogenesis inhibitor) should be affected (28). In vitro studies demonstrated that the TSP-1 homologous protein TSP-2 also binds to the latent TGF-β complex via its WSHW motif, but lacks the RFK motif, which is essential for TGF-β activation (36). This putative role of TSP-2 as a competitive inhibitor of TSP-1-mediated TGF-β activation has not been shown in any in vivo situation (36).

This proof of concept study demonstrates for the first time that gene therapy using a TSP-2-overexpressing plasmid is feasible and able to inhibit TGF-β activation but not expression, as indicated by several different methods, including immunohistochemistry for active or total TGF-β, the signal transduction complex Smad 2/3, and transcription of TGF-β target genes PAI-1 and matrix molecules. These data suggest competition with TSP-1 as the pathophysiological mechanism, since TSP-2 plasma levels were increased, whereas glomerular TSP-1 was unchanged in the TSP-2-overexpressing group. In addition, MC activation and conversion toward the myofibroblast phenotype acquiring de novo expression of α-smooth muscle actin and collagen I is considered an important step in renal fibrosis and dependent on TGF-β and/or other mediators such as MMPs (8, 39). TSP-2 overexpression in nephritis inhibited also the mesangial phenotype switch toward a myofibroblast-like type, which also may relate to inhibition of TGF-β activation. The beneficial effects as demonstrated in this study are comparable with previous studies antagonizing TGF-β by specific antibodies or decorin or TSP-1-blocking peptides or TSP-1 antisense oligonucleotides (12, 13).

Downregulation of MMP-2 is another putative mechanism how TSP-2 can potentially inhibit renal fibrosis. In a recent paper by Cheng et al. (8), overexpression of MMP-2 in renal tubular cells promoted epithelial-mesenchymal transition and interstitial fibrosis (8). TSP-2 downregulates MMP-2 activity.

Fig. 8. TSP-2 overexpression reduced glomerular inflammatory cells in rats with anti-Thy1 disease. Glomerular infiltration with T cells was assessed by analysis of CD3 (A and B, arrows indicate dark gray CD3-positive staining) and CD8 (C and D, fluorescing glomerular CD8-positive cells were marked by arrows)-positive cells per glomerular cross section using immunohistochemistry. Antigen-presenting cells were investigated by immunohistochemical staining for myosin heavy chain (MHC) II (E and F, fluorescing glomerular MHC II-positive cells were marked by arrows). Glomerular macrophages were detected by staining for ED-1 (G and H, arrows indicate dark gray ED-1-positive staining) and B cells by staining for CD45R (I and J, fluorescing glomerular CD45R-positive cell were marked by an arrow). All representative images show glomeruli from anti-Thy1 nephritic rats treated with the lacZ-overexpressing plasmid. *Statistically significant differences between groups treated with gene therapy (P < 0.01).
in vitro by formation of a complex with the zymogen, which inhibits its activation (5), and internalization by an low-density lipoprotein receptor protein 1 with subsequent lysosomal degradation (40). In our study, glomerular MMP-2 activity was significantly decreased in TSP-2-overexpressing nephritic rats on day 3 but not on day 6, indicating an additional MMP-2-dependent mechanism of myofibroblast conversion in this model (besides regulating TGF-β activity). Consistent with this finding, TSP-2 deficiency during experimental glomerulonephritis led to increased MMP-2 activity (10).

The third mechanism how TSP-2 ameliorates anti-Thy1 nephritis is inhibition of glomerular proliferation. In this study, TSP-2 overexpression inhibited both endothelial and mesangial proliferation, leading to reduced excess of glomerular cellularity and size, both of which are important markers of disease. The antiproliferative effect of TSP-2 was shown before, mainly in endothelial cells in vitro (4) and in vivo (10), while our latter study demonstrated antiproliferative activity also for renal tubular cells. In this study, we could demonstrate a direct antiproliferative effect of TSP-2 on MCs in vitro. This finding most likely relates to direct cell cycle regulatory effects as shown in in vitro studies (4) and seems to be independent of TGF-β. TGF-β inhibits cell proliferation in vitro in different cell types, including MC (35); therefore, reduced TGF-β activity should potentially result in excess proliferation. In addition, several in vivo studies either directly blocking TGF-β (2, 6) or blocking TSP-1-mediated TGF-β activation (12, 13) did not find any change in glomerular proliferative activity during the anti-Thy1 model.

An anti-inflammatory action of TSP-2 is the forth mechanism how TSP-2 can exert beneficial effects during experimental glomerulonephritis. Inflammatory cells can activate MC by secreting a variety of cytokines, including platelet-derived growth factor, tumor necrosis factor-α, and TGF-β, resulting in renal fibrosis (15). Furthermore, chemokines are also known inducers of renal fibrosis (3). For example, inhibition of glomerular leukocyte infiltration by FTY720 slows progression of chronic anti-Thy1 nephritis (34). In this study, we demonstrated an anti-inflammatory effect of TSP-2 on CD3-, or CD8-positive, T cells as well as MHCCI class-positive cells but not on B cells or monocytes/macrophages. In accordance with this finding, TSP-2 deficiency led to an accelerated and increased influx of inflammatory cells during anti-GBM nephritis (10) and in delayed-type hypersensitivity reactions elicited in the skin (27). TSP-2 seems to exert its anti-inflammatory action by regulation of proinflammatory mediators. In rheumatoid arthritis, TSP-2 suppressed the production of interferon-γ and tumor necrosis factor-α and induced the depletion of tissue-residing T cells (33). Although no data are available regarding the mechanism by which TSP-2 regulates these mediators, the direct or indirect modulation of the expression or function of chemokines would be consistent with its characteristic features as a matricellular protein (7).

Because thrombospondins can potentially modulate blood pressure by regulation of nitric oxide signaling (21), we also measured this parameter in our study (data not shown). Blood pressure was neither elevated in the nephritis model on day 6 nor altered by TSP-2 gene therapy, indicating that observed changes were not dependent on changes of blood pressure.

Our study has some limitations. First, in this proof of principle study, because of the short time frame of treatment in our glomerulonephritis model, long-term side effects of TSP-2 overexpression cannot be ruled out. Second, the anti-Thy1 model cannot be directly compared with human mesangial proliferative nephropathy, but does mimic many features of human renal disease, such as TGF-β activation, matrix accumulation, myofibroblast conversion, and cellular proliferation.

In conclusion, TSP-2 is a promising therapeutic agent to ameliorate glomerulonephritis by interfering with important pathways of renal disease such as glomerular cell proliferation and activation, influx of inflammatory cells, as well as TGF-β and MMP-2-mediated fibrosis.

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