Thrombospondin-1 is a major activator of TGF-β in fibrotic renal disease in the rat in vivo

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Thrombospondin-1 is a major activator of TGF-β in fibrotic renal disease in the rat in vivo.

Background. Transforming growth factor-β (TGF-β), a profibrotic cytokine involved in many scarring processes, has to be activated extracellularly before it can bind to its receptors. Thrombospondin 1 (TSP1), a multifunctional matricellular glycoprotein, has been identified as an activator of TGF-β in in vitro systems and during mouse postnatal development in vivo. TSP1 is expressed de novo in many inflammatory disease processes, including glomerular disease.

Methods. In this study we investigated whether peptides specifically interfering with the activation process of TGF-β by TSP1 may be able to block activation of TGF-β in an in vivo model of mesangial proliferative glomerulonephritis.

Results. Continuous intravenous infusion of blocking peptide by minipumps significantly reduced expression of active TGF-β in glomeruli on day 7 of disease as indicated by immunohistochemistry, bioassay, and activation of the TGF-β signal transduction pathway, while total TGF-β expression was unchanged. Inhibition of glomerular TGF-β activation was accompanied by a decrease of glomerular extracellular matrix accumulation and proteinuria, but was without effect on mesangial cell proliferation or influx of monocytes/macrophages.

Conclusion. TSP1 is a major endogenous activator of TGF-β in experimental inflammatory glomerular disease. Drugs interfering with the activation of TGF-β by locally produced TSP1 may be considered as a future specific treatment of scarring kidney disease.

Extracellular matrix accumulation is one of the hallmarks of inflammatory diseases in many organ systems, including the kidney, and is the major cause of end-stage renal disease in humans. Mesangial proliferative glomerulonephritis, the most common type of glomerulonephritis in the Western world [1], is characterized by

mesangial cell (MC) proliferation and extracellular matrix expansion [2]. In up to 50% of the patients with mesangial proliferative glomerulonephritis, the disease process eventually progresses to end-stage renal disease because specific treatment is still lacking [3]. Typical features of human mesangial proliferative glomerulonephritis are mimicked by an experimental model in the rat, induced by an antibody against the Thy1-antigen on MC [2].

The role of transforming growth factor-β (TGF-β) as a major profibrotic cytokine in the anti-Thy1 model has been well established [4]. It has been demonstrated that TGF-β1 mRNA and protein are increased in the anti-Thy1 model [5], and that blocking TGF-β1 by injections with a polyclonal anti-TGF-β1 antibody or the proteoglycan decorin, a TGF-β1, -2, and -3 binding protein [7], markedly reduced extracellular matrix accumulation [6]. The results of these studies were confirmed by studies using equivalent gene transfer techniques against TGF-β in the anti-Thy1 model [8, 9]. In contrast, mice transgenic for an active form of TGF-β1 exhibit elevated plasma levels of TGF-β1 and develop progressive renal disease characterized by MC matrix accumulation, interstitial fibrosis, and proteinuria [10]. Transfer of the TGF-β1 gene into glomeruli of normal rats caused an increase in glomerular TGF-β1 protein that was linked to extracellular matrix formation [11]. The potential importance of TGF-β in mediating fibrosis also in human kidney disease has been supported by the widespread link of TGF-β up-regulation and extracellular matrix excess in many different types of human kidney disease [4]. While these studies suggest great benefit from suppression of TGF-β function in fibrotic kidney disease, it has to be considered that TGF-β is a multifunctional cytokine that exhibits other essential functions in mammals. Mice lacking either the TGF-β1, -2, or -3 gene do not survive beyond a few weeks after birth [12–14]. Therefore, accurate regulation of TGF-β function seems to be critical for the health of mammals

and any anti-TGFβ1 therapeutic approach should try to target the local overproduction (-function) of TGF-β as specifically as possible.

One possiblilty to approach this goal could be to control the activation process of locally produced TGF-β. TGF-β is secreted by most cell types as a latent, inactive procytokine complex [15]. The mature TGF-β protein has to be extracellularly released from this procytokine complex to be able to interact with its receptors. While various players/mechanisms such as pH changes, gamma irradiation, reactive oxygen species, plasmin, calpain, cathepsin, or TSP1 have been identified as activating TGF-β under in vitro conditions, it is still unknown how TGF-β is activated in an inflammatory process in vivo [15].

Recent data have suggested the homeotrimeric extracellular matrix protein TSP1 as an activator of TGF-β1 in vitro in different cell systems including MC [16–18], as well as in cell-free systems. It has been demonstrated that TSP1 forms a trimolecular complex with the TGF-β procytokine complex by interacting with the mature TGF-β protein as well as the so-called latency-associated peptide (LAP). Hereby, the hexapeptide AAWSHW from the type I repeat of the TSP1 molecule is required for TSP1 binding to the mature TGF-β protein, allowing interaction of the KRFK amino acid sequence of the TSP1 molecule with the N-terminal LSKL sequence of the LAP [19, 20]. This complex interaction leads to a confirmational change, probably within the LAP, that allows the mature TGF-β protein to bind to its receptors. It has been shown that both the hexapeptide AAWSHW and the LSKL peptide are able to block activation of TGF-β by TSP1. In addition, comparing TSP1 null mice with TGF-β1 null mice, Crawford et al [21] identified TSP1 as a major activator of TGF-β1 in pancreas and lung homeostasis in mice pups in vivo. Organ pathology of TGF-β1 null pups and TSP1 null pups were strikingly similar and could be induced in wild-type pups by intraperitoneal treatment with the LSKL peptide that specifically blocks activation of TGF-β1 by TSP1. Loss of TSP1 expression in TSP1-null mice spontaneously produced inflammatory lung disease [22], and histologic changes in TSP1 null mice reverted toward wild-type by treatment with the TGF-β activating peptide KRFK.

Interestingly, TSP1 expression in vitro is regulated by various cytokines such as platelet-derived growth factor (PDGF), fibroblast growth factor-2 (FGF-2), or TGFβ, and is frequently expressed de novo at sites of inflammation and wound healing [23]. The involvement of TSP1 in the anti-Thy1 model has been currently demonstrated [24].

Therefore, we hypothesized that TSP1 is an endogenous activator of TGF-β in inflammatory kidney disease and investigated whether systemic treatment with either one of two blocking peptides that interfere with TSP1-TGF-β1 binding (LSKL or AAWSHW) is able to sup-
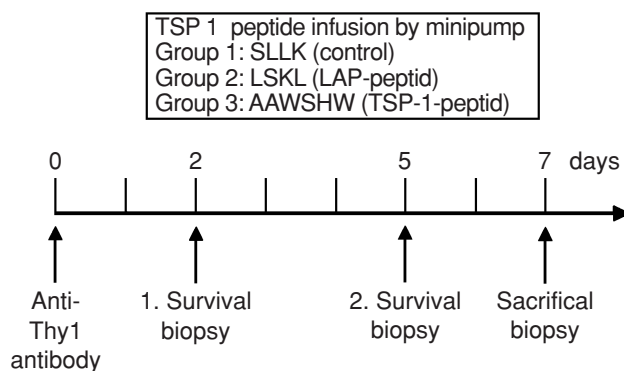

Fig. 1. Shown is the schematic outline of the experimental design.

press activation and thereby function of TGF-β in experimental mesangial proliferative nephritis in the rat.

METHODS

Experimental design

A scheme of the experimental protocol is shown in Figure 1. An identical pilot study in four to six rats per group using half of the peptide dose was done without glomerular preparations. Experimental mesangial proliferative glomerulonephritis was induced using the monoclonal mouse anti-Thy1 antibody OX-7. Peptides were continuously infused intravenously via jugular vein using osmotic minipumps starting 16 hours after disease induction. The effects of the hexapeptide AAWSHW (which interfere with the TSP1-mature TGF-β interaction) or the LSKL peptide (that blocks the TSP1-LAP interaction) were compared with a control peptide (SLLK) in the anti-Thy1 model. Tissues from different time points (days 3, 5, 7) of this experiment were analyzed in regard to mesangiolysis, macrophage influx, MC proliferation, microaneurysm, and matrix formation, GEN proliferation, TSP1, active TGF-β, total TGF-β1 and -2, as well as TGF-βRI and RII, and phosphorylation of the TGF-β signaling molecule Smad2/3. Functional parameters such as blood pressure, proteinuria, and creatinine clearance were also determined. On day 7, glomerular secretion of active TGF-β was determined in individual animals using the NRK bioassay.

Animal model

Experimental mesangial proliferative glomerulonephritis (anti-Thy1 model) was induced in Sprague Dawley rats (180–200 g; Charles River, Sulzfeld, Germany) by a single injection of 1 mg/kg of the mouse monoclonal anti-Thy1 antibody OX-7 (European Collection of Animal Cell Culture, Salisbury, UK). In this animal model, complete anti-Thy1 antibody binding

occurs within an hour [26]. To avoid potential interference of the TSP-peptides with anti-Thy1 antibody binding and subsequent mesangiolysis, the peptide treatment was started 16 hours after disease induction, when binding of the anti-Thy1 antibody to the mesangium and subsequent mesangiolysis had already occurred. The peptides used in this study were synthesized, purified, and analyzed as described elsewhere [27].

As shown in Figure 1, six rats per group received treatment either with a control peptide SLLK-group 1, or with the LAP peptide LSKL-group 2, or with the TSP hexapeptide AAWSHW-group 3. Renal biopsies as described previously [28] were performed on days 3 and 5, and the experiment was finished on day 7. To determine DNA incorporation into proliferating cells, each animal was injected intravenously with BrdU (50 mg/kg bw) 90 minutes before the second survival biopsy was taken on day 5 (peak of MC proliferation). A 24-hour urine collection for measurement of protein and creatinine was done from day 6 to 7, when maximal proteinuria occurs in this model. Blood pressure measurements were done twice before and twice after disease induction. For TGF-β activity measurements in individual animals on day 7, glomeruli were isolated by differential sieving [26] and counted using 5 aliquots. Glomerular isolates were discarded if purity was less than 95%. Glomeruli (8000 mL) were incubated in Dulbecco's modified Eagle's medium (DMEM) at 37°C. After a 24-hour incubation period, glomerular supernatants were stored by −70°C until TGF-β activity measurements were done.

Peptide infusion

All peptides (at a concentration of 7 mg/mL) were continuously infused for 7 days via a catheter in the right jugular vein using osmotic minipumps (Alzet Corp., Charles River, Sulzfeld, Germany). Implantation of minipumps (filling volume: 2 mL, delivery rate: 10 μL/h) and catheter was started 16 hours after disease induction and was immediately followed by an additional intravenous injection of 6 mg peptide per kg body weight before rats recovered from anesthesia. Effective peptide doses were extrapolated from previous pilot studies with TSP peptides as described elsewhere [29], from an additional pilot experiment using these peptides, and from the literature [21, 30]. Because using a peptide concentration of 3.5 mg/mL in this pilot experiment already profound effects on matrix formation and no toxicity were noted, in the final study described in the current paper the peptide dose again doubled compared to the pilot study. The final peptide dose per animal/week in this study (as stated above) is in the same range as used in mice by Crawford et al [21], where the identical peptides were successfully applied intraperitoneally on a daily basis. Considering the results of a study [30] regarding the biodistribution and blood half-life of TSP peptides, a continuous intravenous application of peptides was chosen.

Renal morphology and immunohistochemistry

Renal biopsies were fixed in methyl Carnoy's solution, embedded in paraffin, and cut into 5-μm sections for indirect immunoperoxidase staining as described elsewhere [26]. Sections were also stained with the periodic-acid Schiff reagent and counterstained with hematoxylin. For each biopsy, 40 to 70 cortical glomerular cross-sections were evaluated in a blinded fashion, each containing more than 20 discrete capillary segments.

Mesangiolysis was graded semiquantitatively using the following scale: 0 = no mesangiolysis; I = segmental and focal mesangiolysis (less than 25% of the glomeruli show partial dissolution of the mesangium); II = 25%–50% of the glomeruli are affected; III = most (50%–75%) glomeruli show severe mesangiolysis; and IV = global mesangiolysis, where virtually all glomeruli show a complete dissolution of the mesangial areas. To determine general extracellular matrix formation, sections were also stained with the Masson's Trichrome (blue color) and semiquantitatively scored from 0 to 3 as follows: score 0 = glomerulus without any blue staining; score 1 = glomerulus with little blue staining; score 2 = glomerulus with moderate blue staining; and score 3 = glomerulus almost completely filled with blue staining.

The following antibodies were used in this study: a murine immunoglobulin (Ig)M monoclonal antibody (mAb) against the proliferating cell nuclear antigen (PCNA) (19A2; Coulter Immunology, Hialeah, FL, USA); a murine IgG monoclonal antibody (mAb) against bromodeoxyuridine (BrdU); (ED-1, a murine IgG$_1$ mAb to a cytoplasmic antigen present in monocytes, macrophages, and dendritic cells (Serotec, Ltd., Oxford, UK); OX-7, a murine IgG$_1$ mAb specific for mesangial cells (Serotec); Immunostaining for matrix proteins was conducted with polyclonal antibodies to collagen I, collagen IV (goat anti-human/bovine collagen IV; Southern Biotechnology Associates, Inc., Birmingham, AL, USA), fibronectin (rabbit anti-rat fibronectin; Chemicon International, Inc., Temecula, CA, USA), active TGF-β1 (chicken anti-human active TGF-β1; R&D Systems, Wiesbaden-Nordenstadt, Germany [25], TGF-β1 (rabbit anti-human TGF-β1; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), TGF-β2 (rabbit anti-human TGF-β2; Santa Cruz), TGF-βR1 (rabbit anti-human TGF-βR1; Santa Cruz), TGF-βR2 (rabbit anti-human TGF-βR2; Santa Cruz), Phospho-Smad2/3 (rabbit anti-human Smad2 peptide phosphorylated at Ser-433/435; Santa Cruz), and a murine IgG$_1$ mAb against TSP1 (clone A 6.1; Dunn, Labortechnik GmbH, Asbach, Germany). Negative controls for immunostaining included either deleting the primary antibody or substitution of the primary antibody with equivalent

concentrations of an irrelevant murine monoclonal antibody or preimmune rabbit IgG.

Glomerular active TGF-β and expression of matrix monitored by collagen I, collagen IV, and fibronectin was quantified by computerized measurement of the positive-stained glomerular area using the Metavue Imaging System (Visitron Systems GmbH, Puchheim, Germany) and with equivalent results by a semiquantitative scoring system (data not shown) as described below. Total TGF-β1, total TGF-β2, and TSP1 was graded semiquantitatively [24] and reflected changes in the area and intensity of mesangial staining: 0: very weak or absent staining; 1+: weak staining with <25% of the glomerular tuft showing focally increased staining; 2+: 25%–49% of the glomerular tuft with focally increased staining; 3+: 50%–75% of the glomerular tuft demonstrating increased staining; 4+: >75% of the glomerular tuft stained strongly. In addition, the average number of ED-1 or phospho-Smad2/3 positive cells per glomerular cross-section was determined.

Immunohistochemical double staining

To determine the number of proliferating MC, double immunostaining for PCNA or BrdU, both markers of cell proliferation, and for OX-7, a MC-specific marker was performed as described previously [24]. The number of proliferating MC was evaluated by counting the number of cells that stained for both PCNA (black) and OX-7 (brown) or BrdU (black) and OX-7 (brown) as PCNA+/OX-7+ or BrdU+/OX-7+ cells, respectively, and was expressed as mean ± SD per glomerular cross-section.

Glomerular TGF-β-activity

Active TGF-β was measured three different ways. First, using a well-established bioassay system, active TGF-β present in glomerular supernatants after a 24-hour incubation period was determined by colony formation by NRK cells in soft agar assays as described previously [18]. The number of colonies greater than 62 μm (≥8–10 cells) in diameter was counted. Recombinant active TGF-β1 (R&D Systems, Germany) was used as a control. All experiments were done twice in triplicate. In addition, activation of TGF-β was evaluated using an antibody against active TGF-β1 (chicken anti-human active TGF-β1, R&D Systems, Germany) [25], and an antibody against the phosphorylated form of the TGF-β signaling molecule Smad2/3 by counting positive nuclei per glomerular cross section (Santa Cruz).

Miscellanous measurements

Urinary protein was measured using the BioRad Protein Assay (München, Germany) and bovine serum albumin (BSA) (Sigma, Deisenhofen, Germany) as a standard. Creatinine in serum or urine, as well as blood urea nitrogen, were measured using an autoanalyzer (Beckman Instruments GmbH, München, Germany). Systolic blood pressure was measured by tail plethysmography in conditioned, conscious rats [31].

Statistical analysis

All values are expressed as mean SD. Statistical significance (defined as $P < 0.05$) was evaluated using the Student t test or one-way analysis of variance (ANOVA) with modified t test using the Bonferroni method.

RESULTS

Blocking peptides decreases activation of TGF-β in glomeruli from rats with anti-Thy1 disease

If the de novo expressed TSP1 is activating TGF-β in the anti-Thy1 model, TGF-β activity in glomeruli from blocking peptide treated animals had to be reduced. Since the amount of active TGF-β1 or TGF-β2 in glomeruli from individual rats was too small to be detectable by commercially available TGF-β-Assays (R&D Systems, or Genzyme, Neu-Isenburg, Germany), TGF-β bioassays for measuring the active TGF-β fraction were applied. In the mink lung assay, TSP peptides have been shown to influence growth of the mink lung cells in a TGF-β–independent fashion ([27] and this study). Since the NRK assay has been shown to be sensitive and specific for TGF-β activity measurements [16, 18–21], this well-established assay was used to determine glomerular TGF-β activity of peptide-treated animals. Since detergents used for protein extraction of glomeruli interfer with the NRK bioassay (this study) and may potentially lead to unspecific activation of TGF-β, isolated glomeruli from day 7 animals were incubated for a 24-hour period in DMEM at 37°C, and the amount of secreted active TGF-β by these glomeruli was determined in the supernatant. As shown in Figure 2A, glomerular secretion of active TGF-β was markedly reduced in the LAP peptide (LSKL) treated group as well as in the TSP1 peptide (AAWSHW) treated group compared to control peptide (SLLK) treated rats ($P < 0.01$).

In addition, active TGF-β in nephritic glomeruli was determined by immunostaining with an antibody recognizing the active form of TGF-β1 (Fig. 2C). In agreement with the bioassay results, the treatment with the two blocking peptides, but not with the control peptide, was associated with a markedly decreased glomerular TGF-β activity measured by computerized morphometry (Fig. 2B).

Binding of active TGF-β to its receptors is followed by activation of the Smad signaling pathway. Phosphorylation and transport into the nucleus of Smad2/3 is an important part of the TGF-β–mediated signaling pathway and serves as a marker for TGF-β activation [32]. Therefore, activation of TGF-β was additionally evaluated using an antibody against the phosphorylated form of the Smad2/3 molecule and the ratio of positive nuclei per
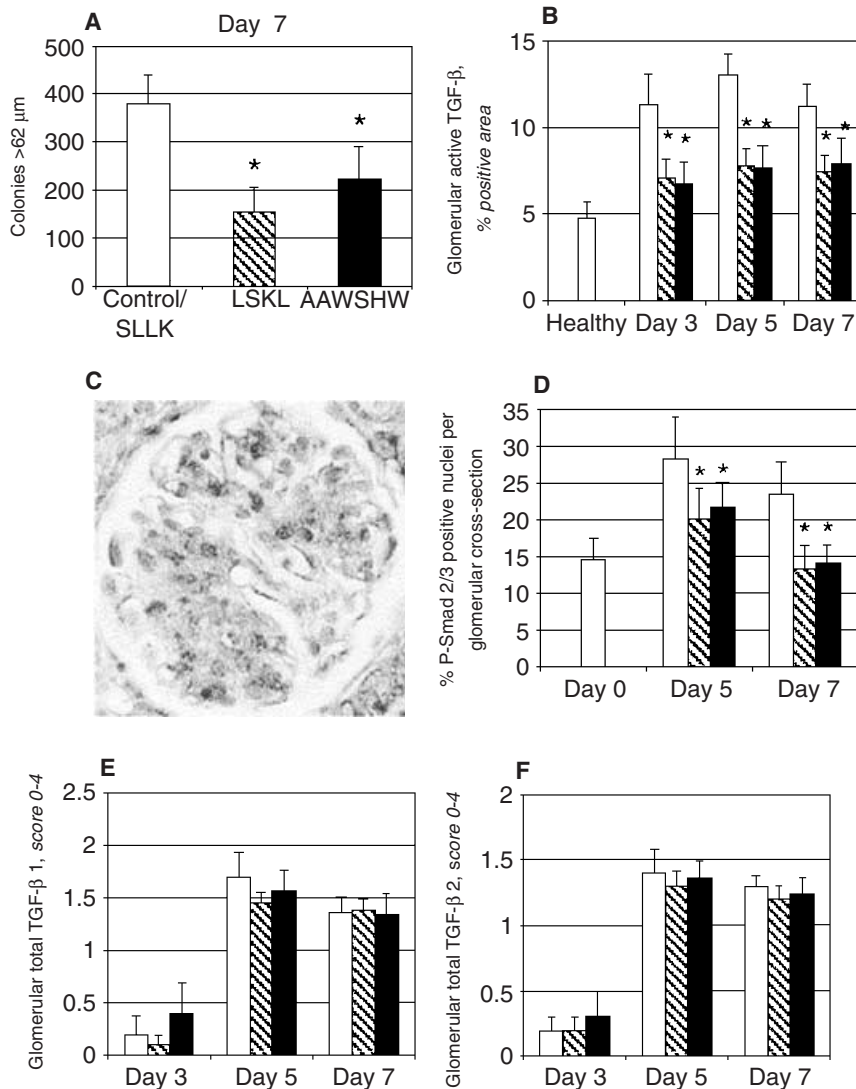
Fig. 2. Therapy using blocking peptides (LSKL or AAWSHW) decreased glomerular activation of TGF-β in rats with anti-Thy1 disease. TGF-β activation was monitored either in supernatants from day 7 isolated glomeruli by using the NRK-bioassay (*A*), by immunostaining of active TGF-β (*B, C*, gray staining), or immunostaining of the phosphorylated TGF-β signaling molecule Smad2/3 in paraffin embedded tissue sections (*D*). A marked reduction of the active fraction of glomerular TGF-β was induced by either blocking (LSKL, hatched bars, or AAWSHW, black bars) versus control (SLLK, white bars) peptide and could be demonstrated by all used methods (A, B, D). In contrast, total TGF-β1 (*E*) or TGF-β2 (*F*) as assessed by immunostaining was not significantly affected by blocking peptid therapy. The asterisk marks significant differences (*P* < 0.01) of the blocking peptide groups versus the control group.

glomerular cross-section was determined. Again, LSKL more than AAWSHW treatment reduced glomerular TGF-β activity on days 5 and 7 compared to the SLLK control as assessed by P-Smad2/3 immunostaining (Fig. 2D). On day 7 of anti-Thy1 disease, blocking peptide treatment led to a complete reduction of the percentage of P-Smad2/3–positive glomerular nuclei down to normal undiseased levels (day 0).

In contrast, total TGF-β1 or TGF-β2 as assessed by immunostaining was not significantly affected by blocking peptide therapy (Fig. 2E and F).

Blocking peptides decreases glomerular extracellular matrix formation in rats with anti-Thy1 disease

Since TGF-β has been demonstrated to cause excess formation of extracellular matrix in the anti-Thy1 model [6–9], we evaluated if suppression of TGF-β activity by blocking peptides is accompanied by decreased matrix formation. Using Masson's Trichrome staining as a general indicator for fibrosis (blue color), no blue staining is detected in normal glomeruli. In the anti-Thy1 model, diseased glomeruli on day 3 or day 5 exhibit no or very little blue staining, while a marked increase in blue staining was seen on day 7 (Fig. 3). Treatment with either the LAP peptide (LSKL) or the TSP1 peptide (AAWSHW) markedly suppressed extracellular matrix formation on day 7 as determined by Trichrome staining (Figs. 3A and B and 4A). This result was confirmed by examining specific typical extracellular matrix proteins such as collagen I and IV, as well as fibronectin during the time course of anti-Thy1 disease using immunohistochemistry. In the anti-Thy1 model, the typical interstitial protein collagen I is transiently expressed de novo by glomerular MC starting on day 3 and peaking on day 5, while the constitutively expressed MC proteins collagen IV and fibronectin are also markedly increased in parallel to collagen I. Treatment with either the LAP peptide (LSKL) or the TSP1
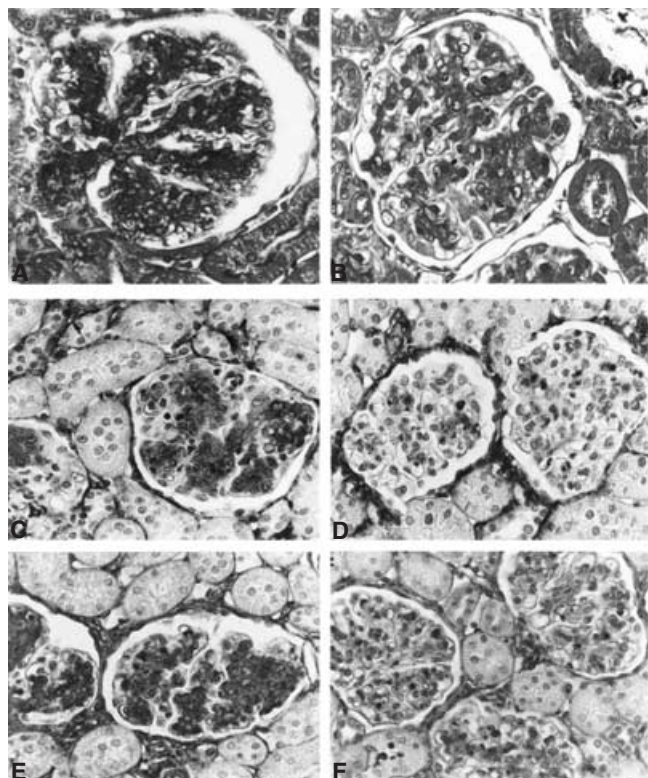
Fig. 3. Blocking peptides (LSKL or AAWSHW) decreased glomerular extracellular matrix formation in rats with anti-Thy1 disease. Using Masson's Trichrome staining as a general indicator for fibrosis (as indicated by dark staining) a marked reduction in blue staining was seen in day 7 glomeruli from the blocking peptide treated groups (*B*) compared to the control group (*A*). In addition, immunostaining for collagen I (*C, D*) or collagen IV (*E, F*) in dark gray was clearly reduced in day 7 glomeruli from the peptide treated animals (*D, F*) compared to control animals (*C, E*).

peptide (AAWSHW) reduced glomerular accumulation of all three extracellular matrix proteins as determined by immunohistochemistry (Figs. 3 and 4). Evaluation of extracellular matrix accumulation using computerized morphometry demonstrated that the changes induced by the treatment of either the LSKL or the AAWSHW peptide were significant compared to the control peptide and that in general the LSKL peptide treatment was slightly superior to the AAWSHW peptide in suppressing matrix accumulation as well as TGF-β activity (Figs. 3 and 4).

Blocking peptides does not affect the glomerular amount of TGF-β1 or -β2, TGFβ -RI or -RII, or TSP1 in rats with anti-Thy1 disease

To examine potential feedback mechanisms between TSP1, active TGF-β, and TGF-β receptors in this model, the amount of glomerular TSP1, TGF-β1 and -β2, as well as TGF-βRI and -RII was evaluated, but no significant changes could be detected comparing the different treatment groups (Table 1, Fig. 2).

Blocking peptides does not affect mesangiolysis, glomerular MC proliferation, or influx of macrophages in rats with anti-Thy1 disease

Mesangiolysis. To ensure that disease induction was equal in all groups, peptide treatment was started 16 hours after anti-Thy1 antibody injection. In addition, mesangiolysis scores on day 3 were equal in all groups (not shown).

MC proliferation. Because TGF-β has been shown to inhibit MC proliferation in vitro [33], we also examined if a reduced TGF-β activity in blocking peptide-treated animals is accompanied by an increased proliferative response of MC. As previously described [24, 28, 29, 34], MC proliferation is already increased on day 3, peaks on day 5, and ceases after day 7. Despite alteration of TGF-β activity and matrix formation, the proliferative response of the MC, as well as the total glomerular cell count were unchanged by any peptide treatment in the anti-Thy1 model (Fig. 5). This result was confirmed by double staining of day 5 biopsies for MC and BrdU, indicating the number of MC that have incorporated the injected BrdU during the phase of DNA synthesis (OX-7+/ BrdU+ cells) (Fig. 5).

Influx of monocytes/macrophages. Since TGF-β has been shown to be chemotactic for monocytes/ macrophages in vitro [35], the number of ED-1–positive monocytes/macrophages per glomerular cross-section was evaluated by immunostaining and did not differ in any group during this experiment (Table 1).

Functional parameters: LSKL peptide infusion decreased proteinuria in experimental glomerulonephritis. Proteinuria, a hallmark of severity of kidney disease, is maximally increased around day 7 in the OX-7 antibody– induced anti-Thy1 model (unpublished observation). Either one of the blocking peptides reduced 24-hour proteinuria on days 6 to 7 compared to control animals; while the effect of the LSKL peptide was more dramatic and did reach significant values, the reduced proteinuria in the AAWSHW-treated rats did not reach significance due to high standard deviation (Fig. 4F). Creatinine clearance on day 7 as a measurement of kidney function tended to be improved by infusion of either one of the blocking peptides, but did not reach significant values (SLLK control 1.52 ± 0.2 mL/min, LSKL 1.78 ± 0.3 mL/min, AAWSHW 1.87 ± 0.2 mL/min). Blood urea nitrogen was also unchanged by the peptide treatment (SLLK control 33.2 ± 12.7 mg/mL, LSKL 33.2 ± 9.4 mg/mL, AAWSHW 23.6 ± 7.1 mg/mL). In addition, none of the blocking peptides affected systolic blood pressure levels in diseased or healthy rats (not shown).

DISCUSSION

Overproduction of TGF-β in response to injury is thought to cause tissue fibrosis in many different
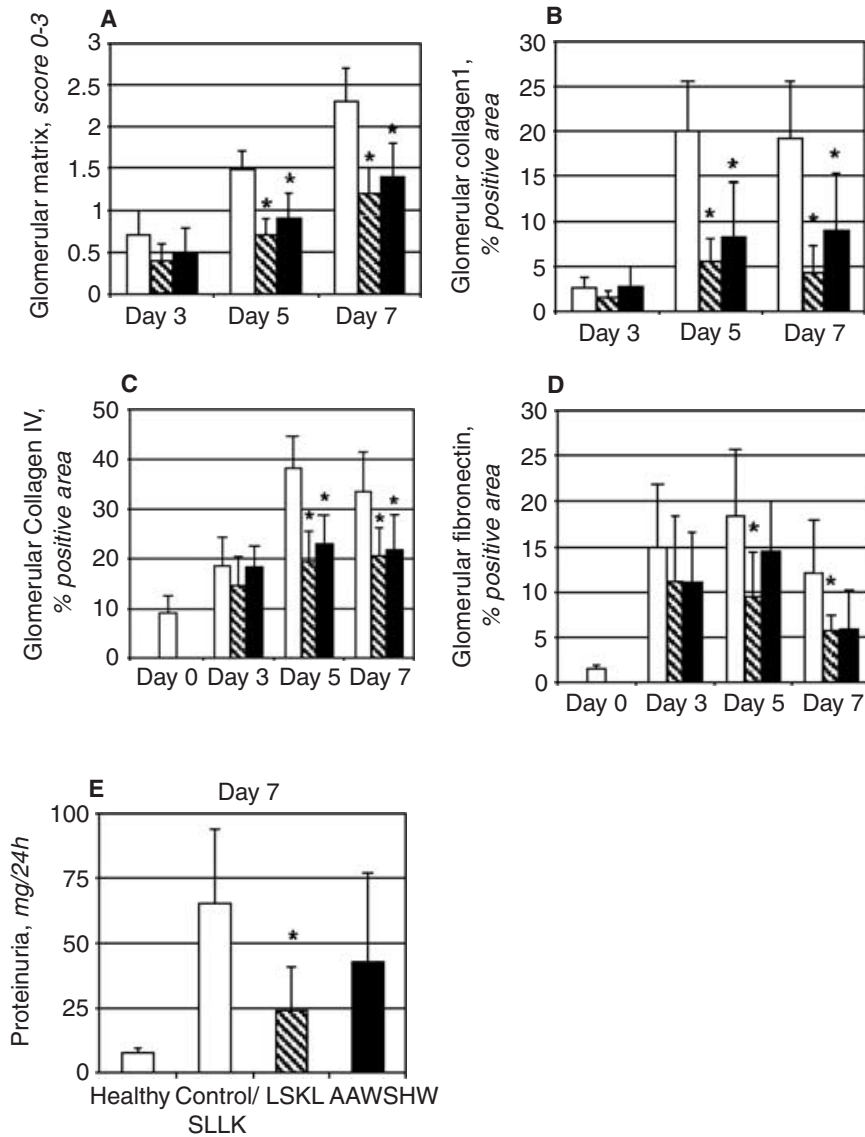
Fig. 4. Blocking peptides decreased glomerular extracellular matrix formation and proteinuria in rats with anti-Thy1 disease. Treatment with blocking peptides (LSKL, hatched bars; AAWSHW, black bars) significantly reduced extracellular matrix formation compared to control peptide (SLLK, white bars), as evaluated by semiquantitative scoring of Masson's Trichrome staining (*A*), or computerized image quantification of immunostaining for collagen I (*B*), collagen IV (*C*), and fibronectin (*D*) during the time course of the anti-Thy1 model. Proteinuria was detected in urine samples collected on day 6/7 on a 24-hour period and demonstrated a significant reduction in the LSKL (hatched bars), but not AAWSHW (black bars) treated versus the SLLK control group (white bars) (*E*). The asterisk marks significant differences ($P < 0.01$) of the blocking peptide groups versus the control group.

inflammatory disease processes. This concept is particularly well established in experimental glomerular disease [4]. By most cells, TGF-β is secreted as a latent procytokine complex that requires extracellular activation before it can interact with its receptors [15]. Despite great interest in therapeutic anti-TGF-β strategies to treat fibrotic disease, the mechanism of TGF-β activation in any inflammatory renal disease in vivo is still unknown**.** The data presented above demonstrate that TSP1 is an important endogenous activator of TGF-β in inflammatory kidney disease. Continuous systemic administration of synthetic peptides inhibited glomerular TGF-β activation by TSP1 in rats with experimental mesangial proliferative glomerulonephritis and provided a remarkable reduction of glomerular extracellular matrix accumulation and proteinuria, while MC proliferation or influx of monocytes/macrophages was not affected.

In the anti-Thy1 model of mesangial proliferative glomerulonephritis, a marked transient de novo expression of the matricellular protein TSP1 by MC (peak on day 5) was regulated by FGF-2 and PDGF [24] and coincided with the up-regulation of TGF-β [5]. The data of the current study demonstrate TSP1 as a major endogenous activator of TGF-β in inflammatory kidney disease and identify a potential therapy for disorders with overproduction (-activation) of TGF-β. Continuous systemic infusion of a peptide (AAWSHW) that inhibits interaction of TSP1 with the mature TGF-β protein (within the TGF-β procytokine complex) [19] or infusion of a peptide (LSKL) that blocks interaction of TSP1 with the LAP of TGF-β was able to reduce the amount of active TGF-β in glomeruli as assessed in three independent ways [20]. This inhibition of glomerular TGF-β activation was accompanied by a marked suppression of the glomerular

Table 1. TGF-β receptor expression, glomerular TSP-1, and glomerular macrophages are not affected by infusion of blocking peptides in glomeruli after induction of the anti-Thy-1 model at days 3, 5, and 7

	Treatment	Day 3	Day 5	Day 7
Glomerular TGF-βRI (Score 0–4)	Control/SLLK	0.4 ± 0.3	2.0 ± 0.5	1.3 ± 0.9
	LSKL	0.2 ± 0.1	1.3 ± 03	0.9 ± 0.6
	rv-amAAWSHWac	0.5 ± 0.5	1.4 ± 0.4	1.2 ± 0.5
Glomerular TGF-βRII (Score 0–4)	Control/SLLK	0.4 ± 0,2	1.9 ± 0.5	1.8 ± 1.0
	LSKL	0.2 ± 0.2	1.3 ± 0.3	1.9 ± 0.4
	rv-amAAWSHWac	0.5 ± 0.4	1.9 ± 0.4	1.6 ± 0.6
Glomerular TSP 1 (Score 0–4)	Control/SLLK	0.2 ± 0.2	1.9 ± 0.7	0.6 ± 0.3
	LSKL	0.3 ± 0.2	1.2 ± 0.5	0.4 ± 0.3
	rv-amAAWSHWac	0.7 ± 0.6	1.6 ± 0.7	0.8 ± 0.7
Glomerular macrophages (ED-1+ cells/ glomerular cross-section)	control/SLLK	4.5 ± 1.5	1.9 ± 0.5	1.5 ± 0.4
	LSKL	4.3 ± 1.9	1.0 ± 0.6	0.8 ± 0.5
	rv-amAAWSHWac	3 ± 1.7	1.0 ± 0.5	0.7 ± 0.2

matrix excess. The LSKL peptide was slightly superior in inhibiting activation of TGF-β and extracellular matrix accumulation. In addition, the LSKL peptide treatment also significantly reduced proteinuria, a hallmark of severity of kidney disease, while the reduction of proteinuria by the AAWSHW peptide did not reach significant values. Both therapeutic effects of the peptide treatment, suppression of extracellular matrix accumulation and proteinuria, are in good agreement with previous studies antagonizing TGF-β by antibodies, decorin injections, or gene therapy [6–11].

Excessive proliferation of glomerular cells is characteristic for many renal diseases and is frequently linked to extracellular matrix accumulation [2]. Although TGF-β inhibits cell proliferation in vitro in different cell types including MC [33], the pathophysiologic role of TGF-β in regard to mesangial cell proliferation in vivo is still controversely discussed. In vivo transfection of the TGF-β1 gene into glomeruli of normal rats induced glomerular hypercellularity [11], while transfer of the TGF-β1 gene into nephritic glomeruli during anti-Thy1 disease [37] led to a reduced glomerular mitogenic activity as determined by ^{3}H-thymidine incorporation. Other studies in the anti-Thy1 model inhibiting TGF-β1 did not find any affect on glomerular cell number [8], or did not evaluate glomerular cell proliferation [6, 7, 9]. Our study now demonstrates that the TSP1-mediated activation of TGF-β is not influencing MC proliferation in experimental mesangial proliferative glomerulonephritis at any time point.

Influx of monocytes/macrophages into the glomerulus is also a characteristic feature of inflammatory glomerular disease. While in vivo and in vitro studies have shown that TGF-β1 can be chemotactic for mononuclear cells as well as it can reduce macrophage adhesiveness potentially leading to deactivation and/or increased clearance from inflammatory sites [38], blocking peptide treatment in this study did not affect glomerular macrophage accumulation in the anti-Thy1 model.

Because TSP1 is able to activate both TGF-β1 and TGF-β2 in an identical manner that can be blocked by either peptide [20], and because glomerular TGF-β1 and TGF-β2 are increased in the anti-Thy1 model ([39] and this study), it cannot be excluded that the effect of the blocking peptide treatment is caused by inactivation of both TGF-β1 and TGF-β2. Nevertheless, the therapeutic effect seen by TGF-β1 inhibition using polyclonal antibodies or antisense oligonucleotides was very similar to the effects of the blocking peptide treatment in this study [6, 8].

Comparing the degree of glomerular TGF-β activation and extracellular matrix formation in blocking peptide-treated diseased animals to normal rats, our data suggest that TSP1 is the major activator of TGF-β in this model. The specific inhibition of TSP1-mediated TGF-β activation is consistent with the role of TSP1 in regard to TGF-β activation during mouse postnatal development, and may prove to be a great advantage as a potential anti-TGF-β strategy in inflammatory disease as supported by studies comparing the TSP1 and TGF-β1 null mice during mouse development [21]. Pathologic changes in several organs of the TSP1 null pups are caused by a reduced, but not completely lacking activity of TGF-β, and the very severe phenotype of the TGF-β1 null mice leading to early death in a generalized excessive inflammatory response [12] is not resembled by the TSP1 null mice [21]. In addition, mice with deletion of only one allele of TGF-β1 have generally reduced TGF-β1 serum and tissue levels, which is associated with increased cell turnover and susceptibility to tumorigenesis in liver and lung [40], a finding that has not been described for the TSP1 null mice. Therefore, therapeutic strategies focusing on nonspecific, systemic blockade of TGF-β ligand-receptor interactions may have a problematic side effect profile considering the complex function of TGF-β in vivo. In contrast, targeting TSP1-mediated activation of TGF-β as a therapeutic intervention for fibrotic kidney disease may have great promise because alternate activation pathways of TGF-β for other functions are not affected. Specificity of this treatment relates to the fact that TSP1-mediated TGF-β activation requires a direct interaction of secreted TSP1 and TGF-β in a complex extracellular neighborhood, and that TSP1 is tightly regulated in disease. In most in vitro systems or in normal tissues very little TGF-β is present in its biologically active form. In contrast, the latent TGF-β procytokine complexes and the TGF-β receptors are highly and widely expressed in most tissues. In this context, it is interesting that in vivo gene transfer of the constitutively active TGF-β1 gene into the lung of rats caused extensive fibrosis, while overexpression of the latent TGF-β1 transgene did not [41]. Although TGF-β is also up-regulated in many disease processes including the anti-Thy1 model, the studies described above and the
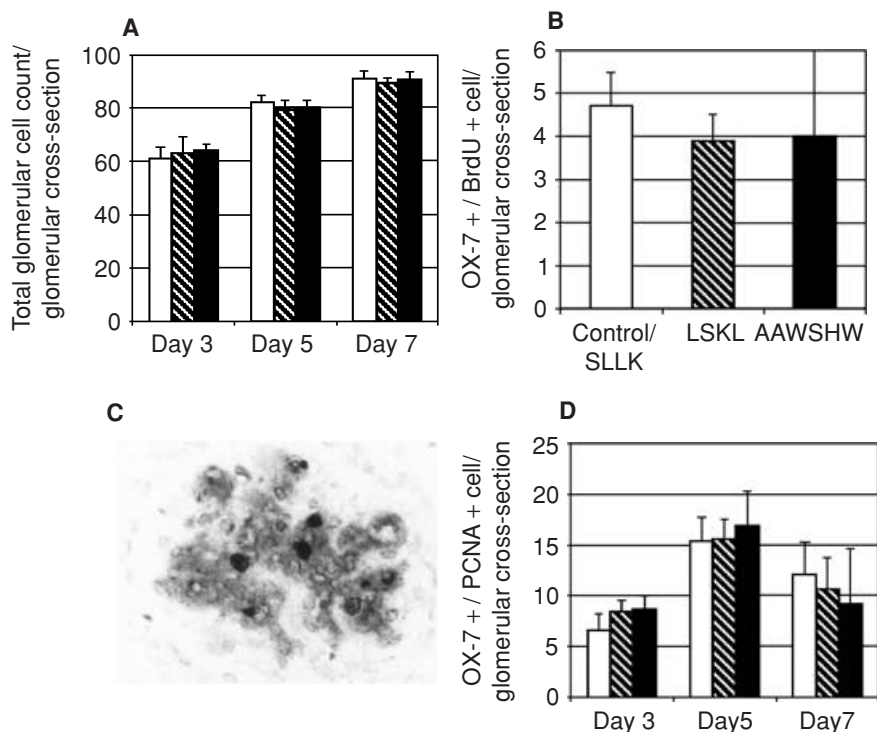
Fig. 5. Blocking peptides did not affect glomerular mesangial cell (MC) proliferation. No significant differences of the blocking peptide groups (LSKL, hatched bars; AAWSHW, black bars) versus the control group (SLLK, white bars) could be detected regarding the number of cells per glomerular cross section (*A*) or the number of proliferating MC as assessed by double immunostaining for MC (anti-OX-7 antibody, gray staining) and either incorporated BrdU (black staining) as a marker for cell proliferation at the day 5 biopsy (*B, C*) or proliferating cell nuclear antigen (PCNA) (*D*) at all days.

data from this study suggest that regulation of TGF-β activation and therefore, of a TGF-β activator, is critical to its profibrotic action. TSP1 perfectly fits into the role of a tightly regulated activator of TGF-β that is induced by other cytokines such as PDGF and FGF-2, as well as potentially TGF-β in response to injury. While in the normal rat glomerulus, glomerular TSP1 expression is below detection level, in anti-Thy1 disease it is transiently dramatically up-regulated by PDGF and FGF-2 [24] in parallel to TGF-β.

Because human renal diseases such as IgA nephropathy usually progress over many years, continuous intravenous infusion of peptides is certainly not a therapeutic option for slowly progressing human renal diseases. We consider this study a "proof of principle study," demonstrating for the first time that blocking TSP1-TGF-β interaction should be a future goal for developing drugs (peptidomimetics) in renal disease. Structural studies are ongoing to attempt to design such compounds. Although the anti-Thy1 model cannot be directly compared with human mesangial proliferative nephropathy, it is a renal disease model mimicking many features of human renal disease, and where renal matrix accumulation is clearly shown to be TGF-β–dependent. In many injury models in different organs as well as in human kidney disease [37, 43], TSP1 expression is consistent with a role of TSP1 in mediating TGF-β activation and possibly fibrosis. Therefore, while the pathogenetic role of this mechanism has to be proven for each of these disease processes, this work does define an important and novel molecular target that

may potentially apply to many different situations with fibrosis.

CONCLUSION

The studies described above identify TSP1 as a major activator of TGF-β in an inflammatory glomerulonephritis model in the rat. This activator of TGF-β is tightly regulated by cytokines in response to injury in this model. The interaction of TSP1 with TGF-β is responsible for most of the glomerular matrix formation occurring in this model, but does not appear to influence MC proliferation or macrophage accumulation. The link of TSP1 and TGF-β in several experimental kidney disease models, as well as the widespread up-regulation of TSP1 in experimental inflammatory processes in other organs as well as in human disease, suggests a central role of TSP1 in mediating tissue fibrosis through interaction with latent TGF-β. A therapeutic strategy inhibiting specifically only the TSP1-mediated TGF-β activation in inflammatory disease may prove to be especially favorable given the known dual effects of TGF-β as a profibrotic as well as an anti-inflammatory cytokine.

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