Thrombospondin-1 is an endogenous activator of TGF-beta in experimental diabetic nephropathy in vivo.

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Running title: Thrombospondin-1 activates TGF-ß in diabetic nephropathy

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Abstract

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

Research design and methods: Type I diabetes was induced in wild-type (n= 27) and TSP-1 deficient mice (n= 36) via streptozotocin injection and diabetic nephropathy was investigated after 7, 9.5 and 20 weeks. Renal histology, TGF-ß activation, matrix accumulation and inflammation were assessed by immunohistology. Expression of fibronectin and TGF-ß was evaluated using Real-time PCR. Furthermore, functional parameters were examined.

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

Conclusion: We conclude that TSP-1 is an important activator of TGF-ß in diabetic nephropathy in vivo. TSP-1 blocking therapies may be considered a promising future treatment option for diabetic nephropathy.
Introduction

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

TGF-β is secreted as an inactive pro-cytokine complex that consists of the mature, active TGF-β protein non-covalently bound to a dimer of its N-terminal propeptide, the so-called latency-associated protein (LAP), and variably to a latent TGF-β binding protein (LTBP). Before it can bind to its receptors, TGF-β must to be activated extracellularly (7). Exposure of TGF-β to pH-changes, gamma irradiation, reactive oxygen species, plasmin, calpain, cathepsin, some integrins or TSP-1 activates TGF-β under in vitro conditions (7). The trimeric TSP-1 can activate TGF-β by binding to both the latency associated protein and the mature TGF-β. This complex interaction reportedly leads to a conformational change within the LAP that allows the mature TGF-β protein to bind to its receptors (8; 9). There is strong evidence suggesting a potential key role of TSP-1 in TGF-β activation and pathogenesis during diabetic nephropathy. First, induction of TSP-1 by high glucose is reported for different renal cell types such as isolated mesangial cells (10; 11), proximal tubule cells (12) and distal tubule cells (13) in vitro. Up-regulation of TSP-1 expression by high glucose is regulated by altering cGMP-dependent protein kinase activity (14; 15). Second, all of the above mentioned studies demonstrated that TSP-1 is a major activator of TGF-β in vitro (10-14). Third, up-regulation of both TGF-β and TSP-1 was also confirmed during diabetic nephropathy in man (16), indicating a potential role of TSP-1 for the activation of TGF-β in human diabetic nephropathy. Furthermore, our group demonstrated that TSP-1 is the major activator of TGF-β in an experimental mesangial proliferative glomerulonephritis in rat by two independent methods (17; 18). Nevertheless, the role of TSP-1 in experimental diabetic nephropathy in vivo is still unknown. Therefore, we hypothesized that TSP-1 is an endogenous activator of TGF-β in diabetic nephropathy and investigated whether TSP-1 deficiency is able to suppress activation and therefore function of TGF-β in streptozotocin induced diabetic nephropathy in the mouse.

Methods

Animals.

The animal studies were performed in accordance with the internal animal review board (Regierung von Mittelfranken: 621-2531.31-17/05). The animals were housed in a room maintained at 22 ± 2°C and exposed to a 12 : 12h dark light cycle and fed standard mouse chow (Altromin 1324, Spezialfutterwerke GmbH, Lage, Germany) and tap water ad libitum. This study was performed in two different background strains comparing TSP-1 deficient with wt mice as previously described (19) to robustly test our hypothesis of TSP-1 as an important mediator of TGF-β activation during experimental diabetic nephropathy. In
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The first part of the study (7 and 9.5 weeks of diabetes) mice of C57Bl6 background (8 generations backcrossed) and in the second part (20 weeks of diabetes) mice of 129SVJ background were used.

Experimental design. A scheme of the experimental protocol including group sizes is shown in Figure 1. Experimental Type 1 diabetes was induced by a single intraperitoneal injection of 180 mg streptozotozin (STZ, Sigma, St. Louis, Miss., USA) per kg bodyweight injected in 50 mM sodium citrate buffer pH 5.5 using mice of 3 month age. Successful diabetes induction was first tested one week after induction followed by monthly blood glucose measurements. Non-diabetic control animals were treated with solvent alone. At least seven mice per group with repetitively confirmed high glucose levels (>400 mg/ml) were sacrificed 7, 9.5 and 20 weeks after induction of diabetes. During the long term (20 weeks) diabetes experiment we transiently administered long-acting insulin (Lantus, human insulin HI-901; Sanofi Aventis, Frankfurt, Germany) subcutaneously at a dose of 2 units whenever the condition of animals was impaired or weight loss occurred. Using this procedure as described before (20-22), we successfully rendered animals hyperglycemic without becoming ketoacidotic. The day before sacrifice, a 24h urine collection was performed and proteinuria, albuminuria as well as creatinine clearance was determined. On the day of sacrifice, blood was collected via vena cava puncture and kidneys were perfused with 0.9% sodium chloride followed by perfusion with Deltadex 40 including 0.05% Novocain and the right kidney was harvested for immunohistochemical analysis. Afterwards, the animals underwent perfusion fixation with 48mM Na₂HPO₄ and 14mM KH₂PO₄ including 3% glutaraldehyd for preparation of semi-thin sections (only done for the 20 week diabetes time point).

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

To perform immunostaining, tissue sections were incubated with the following primary and secondary antibodies: 19A2, a murine IgG monoclonal antibody (mAb) against the proliferating cell nuclear antigen (PCNA; Chemicon, Temecula, CA, USA,(24)) indicating actively proliferating cells; a rat monoclonal IgG2a to mouse CD4 antigen (Caltag Lab., Burlingame, CA, USA); a rat monoclonal IgG2a to mouse CD8a antigen (Caltag Lab.,(24)); F4/80, a murine IgG1 mAb to a surface receptor present on monocytes, macrophages, and dendritic cells (Caltag Lab.); a polyclonal antibody to human collagen IV (Southern Biotechnology Associates, Birmingham, UK); fibronectin, a rabbit polyclonal antibody to rat fibronectin (Chemicon) (18)); a murine IgG₃ mAb against TSP-1 (Dunn, Asbach, Germany,(25)), a murine monoclonal antibody for human desmin (DAKO, Glostrup, Denmark, (26)); a rabbit polyclonal antibody to
phosphorylated Smad2/3 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA,(17)); a chicken polyclonal anti-human active TGF-ß1 (R&D systems, Germany, (18)); a rabbit polyclonal to human plasminogen activator inhibitor-1 (Santa Cruz). Negative controls for immunostaining included either the omission of the primary antibody or substitution of the primary antibody with equivalent concentrations of an irrelevant murine mAb or pre-immune rabbit IgG. All immunoperoxidase stainings was done using methyl Carnoy's-fixed tissues. After incubation with primary antibodies overnight at 4°C, specific biotinylated secondary antibodies (all by Vector Lab., Burlingame, CA, USA) were applied to tissue sections, followed by peroxidase-conjugated Avidin D (Vector), and color development with diaminobenzidine, with or without nickel chloride, for nuclear staining.

For frozen sections a Cy3-labeled donkey anti-rat IgG (Jackson Lab, West Grove, PA, USA) was used as secondary antibody. For each analysis, 25 to 50 glomerular cross-sections were evaluated in a blinded fashion. Glomerular active TGF-ß and expression of fibronectin was quantified by computerized measurement of the positive stained glomerular area using the Metavue Imaging System (Visitron Systems GmbH, Puchheim, Germany). Mesangial matrix expansion was either graded on a scale of 0 to 4 (0 = normal/no staining, 1 = less than 25%, 2 = 26% - 50%, 3 = 51% - 75%, and 4 = > 75% of the glomerulus involved or evaluated in semi-thin sections using a 10x10 grid overlaying each glomerulus and counting the number of squares completely filled out by PAS positive mesangium. Collagen IV was graded semi-quantitatively 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, glomerular PAI-1 expression was also graded semi-quantitatively on a scale of 0 to 4 (0 = absent staining, 1 = weak podocytic staining, 2 = strong podocytic staining, 3 = podocytic and mesangial staining, 4 = podocytic and mesangial staining of more than 50% of the glomerular tuft). For slides immunostained for desmin, only the podocytes at the outer edge of the glomerular tuft were assessed as described elsewhere (26). F4/80+, CD4+ and CD8a+ cells were analysed in 20 fields (x400 magnification).

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

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

Real-time RT-PCR was performed on a TaqMan® ABI 7000 Sequence detection system using the Mastermix (all Applied Biosystems). After an initial hold of 3 minutes 95°C samples were cycled 40 times at 95°C for 15 seconds.
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and 60°C for 60 seconds. The cDNA content of each sample was compared with 18S (forward primer: 5'-TTGATTAAGTCCCTGCCCTTTGT-3'; reverse primer: 5'-CGATCCGAGGGCCTCACTA-3'; (43)) as a housekeeping gene following the ∆∆Ct technique. For Real-time PCR the following primers were used: fibronectin (forward primer: 5'-TGTGACCAGCAACACGGTG-3'; reverse primer: 5'-ACAAACAGGAGTAGGGCGC-3'); TGF-ß1 (forward primer: 5'-TGACGTCACTGGAGTTGTACGG-3'; reverse primer: 5'-GGTTCATGTCATGGATGGTGC-3'; probe: 5'-TTCAGCGCTCACTGCTCTTGTGACGG-3' (42)); TGF-ß2 (purchased from Qiagen; QuantiTech Primer Assay, Hilden, Germany).

Miscellaneous measurements. Urinary protein was measured using the BioRad Protein Assay (München, Germany) with BSA (Sigma, Deisenhofen, Germany) as a standard. Creatinine in serum or urine as well as blood urea nitrogen were measured using an autoanalyzer (Beckman GmbH, Munich, Germany). Albuminuria was determined using an ELISA-Kit (Axxora GmbH, Grünberg, Germany) following the manufactures instructions.

Statistical analysis. All values are expressed as mean ± SD. Statistical significance (defined as p < 0.05) was evaluated using the Student’s t test or one way analysis of variance with modified t test using the Bonferroni method.

Results

TSP-1 is expressed during streptozotocin induced diabetic nephropathy in mice

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

TSP-1 deficiency reduces TGF-ß activation without changing total TGF-ß in diabetic nephropathy

If the de novo expressed TSP-1 is activating TGF-ß in STZ-induced diabetic nephropathy, glomerular TGF-ß activity, but not total TGF-ß expression should be reduced in TSP-1 deficient mice. Active TGF-ß in glomeruli injured by diabetes was determined by using several different read outs: First, immunostaining using an antibody specifically recognizing the active form of TGF-ß1 was performed (Fig. 3A, B). Second, immunostaining with an antibody specific for the phosphorylated form of the TGF-ß signal transduction molecules Smad 2/3 was done (Fig. 3C, D). Finally, staining for the TGF-ß target gene plasminogen activator inhibitor 1 (PAI-1) (Fig. 3E, F) was done. The amount of active TGF-ß steadily increased with duration of diabetes in wt mice reaching highest active TGF-ß
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amounts on week 20 (Fig. 3A). TSP-1 deficiency was associated with inhibition of increased glomerular TGF-β activity 9.5 and 20 weeks after induction of diabetes (Fig. 3A, p < 0.0005). Consequently, the increased percentage of glomerular cells positive for the phosphorylated form of the TGF-β signaling molecule Smad2/3 was substantially lower in TSP-1 deficient diabetic mice at all time points investigated (Fig. 3D, p < 0.0005). TSP-1 deficient mice also showed significantly lower glomerular PAI-1 expression at all time points after diabetes induction (Fig. 3C, p <0.02). In contrast, total glomerular TGF-β was similar in wt and TSP-1 deficient diabetic mice, as assessed by immunostaining for TGF-β 1 and 2 on cross sections (Fig. 4A, B) and by real-time PCR for TGF-β1 and 2 mRNA from microdissected glomeruli 20 weeks after diabetes induction (Fig. 4C, D). Western-blot analysis for TGF-β1 and TGF-β2 using cortical extracts from mice after 9.5 weeks of diabetes (data not shown) also showed increased levels associated with diabetic nephropathy but no differences in wt versus TSP-1 deficient mice.

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

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

Influx of inflammatory cells was reduced in diabetic mice lacking TSP-1
Inflammatory cells are important mediators for the induction of renal fibrosis. Therefore, influx of macrophages and T-cells were investigated in wt and TSP-1 deficient mice during STZ-induced diabetic nephropathy. All inflammatory cell types assessed (macrophages via F4/80
staining, T-cells via CD4- and CD-8 staining) were increased within the renal cortex during diabetic nephropathy. Accumulation of these different inflammatory cell types within the renal cortex was overall lower in TSP-1 deficient mice reaching statistical significance at 9.5 weeks (C57Bl6 background) and variably at 20 weeks (129 SVJ background) after diabetes induction. Accumulation of cortical macrophages was significantly lower in diabetic TSP-1 deficient mice at 9.5 but not 20 weeks after diabetes induction due to a high standard deviation (Fig. 6A). Cortical influx of T-cells as assessed by CD4 and CD8a positivity was significantly reduced by 52% and 64% in TSP-1 deficient mice 9.5 weeks after diabetes induction compared to wt controls (Fig. 6B, C). In parallel to cortical macrophages, T-cell infiltration was more than 4-times higher compared to healthy controls in wt mice after 20 weeks of diabetes. At that time point, significantly lower CD4 positive cells were again found in TSP-1 deficient mice but CD8a-positive cells only showed a tendency to lower numbers (Fig. 6B, C) compared to wt diabetic mice.

### TSP-1 deficiency reduced glomerular cell number in mice 20 weeks diabetic

Since TSP-1 is known to influence both, cellular proliferation and apoptosis, these measures were investigated during diabetic nephropathy. During the first ten weeks of diabetes, glomerular cell number increased only slightly and to an equal extent in wt and TSP-1 deficient mice (C57bl6 background). 20 weeks after diabetes induction (129SVJ background), a significant increase of glomerular cell number was detected in wt mice, which was ameliorated in TSP-1 deficient mice (Fig. 7A, p < 0.0002). Differences in cell number are either due to the balance of local proliferative and apoptotic activity as well as influx of systemic cell types. Glomerular proliferation was slightly enhanced during diabetes in both groups starting on different levels in the two different backgrounds. Independent of the genetic background, neither the proliferative (Fig. 7B) nor the apoptotic activity (as assessed by TUNEL-assay, Fig. 7C, D) within glomeruli or cortical areas was significantly different in TSP-1 deficient versus wt mice.

### Kidney function was improved in diabetic mice lacking TSP-1

Proteinuria, a marker of severity of diabetic nephropathy, was unchanged during early diabetes in both groups indicating only a minor impairment of renal function at the beginning of diabetic nephropathy. Twenty weeks after diabetes induction, proteinuria in diabetic mice was increased by approximately 8-fold as compared to healthy controls. This response was decreased by approximately 50% in TSP-1 deficient mice as compared to wt animals (Fig. 8A, p <0.003). Albuminuria, an even more sensitive marker of renal diabetic injury, was increased during early diabetes in the C57bl6 background (weeks 7-9.5) and was maximally increased 20 weeks after diabetes induction in the 129 SVJ background (Fig. 8B). TSP-1 deficient mice showed a tendency to lower albuminuria levels during early diabetes (7-9.5 weeks), which almost reached significance at week 20 (p = 0.08, Fig. 8B). Serum urea as another indicator of renal functional impairment was also increased during diabetic nephropathy. Serum urea was significantly reduced in TSP-1 deficient mice 9.5 weeks after diabetes induction and showed an almost significant tendency to lower levels 20 weeks after diabetes induction (Fig. 8C, p= 0.06). Creatinine clearance was the same in both groups (9.12 ± 2.8 in wt mice vs. 8.26 ± 3.4 ml/kg body weight in TSP-1 deficient mice), but only
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Discussion

Diabetic nephropathy already is the leading cause for end stage renal disease in the western world (1; 2; 4) and the incidence is increasing. Many in vitro and in vivo studies confirmed TGF-β as a key player in the pathogenesis of diabetic nephropathy (6).

This study, for the first time, shows that TSP-1 is an endogenous activator for TGF-β in diabetic nephropathy in vivo. Deficiency for TSP-1 in diabetic mice reduced activation but not expression of TGF-β in our study. While glomerular levels of TGF-β1 and TGF-β2 protein and mRNA of diabetic wt and TSP-1 ko mice were equivalent, TGF-β activation was reduced in diabetic TSP-1 ko mice as indicated by several different methods for assessing TGF-β activity directly (specific antibody) or indirectly, via the degree of phosphorylation of the TGF-β signaling molecule smad-2/3, as well as the product of the transcriptional TGF-β target, the plasminogen activator inhibitor-1. Reduction of TSP-1 mediated TGF-β activation in diabetic nephropathy apparently is able to ameliorate several key features typical of the pathophysiology of diabetic nephropathy such as increased matrix accumulation and glomerulosclerosis as well as podocyte injury, glomerular hypertrophy and proteinuria.

The data presented here suggest that TSP-1 is an important activator of TGF-β in the STZ-induced diabetic nephropathy model. This interpretation is supported by the fact that some diabetic changes (matrix accumulation) in diabetic TSP-1 ko mice are close to healthy controls and the fact that the pattern and the degree of inhibition of diabetic nephropathy features are very similar to studies using general TGF-β blocking strategies (antibodies) in short term type 1 (29) or long term type 2 experimental diabetic nephropathy (30).

Several recent studies demonstrate the importance of inflammation during diabetic nephropathy (31-33). Therefore, the finding of this study that influx of several different inflammatory cell types, such as macrophages, CD4 and CD8α positive cells, is ameliorated in TSP-1 deficient diabetic mice, as compared to wt mice, suggests that a decreased inflammatory response is the cause of the protective effect of TSP-1 deficiency. It is unclear if this effect is due to altered active TGF-β levels or due to other TSP-1 dependent mechanisms. As discussed in the literature, the role of TGF-β on inflammation is controversial. TGF-β has anti-inflammatory properties as demonstrated by several experimental studies in different models (34; 35) and the fact that TGF-β1 deficient mice die due to multifocal inflammatory disease (36), while TGF-β overexpressing mice exert beneficial anti-inflammatory actions of TGF-β (37). On the other hand, TGF-β can induce macrophage chemotaxant protein 1 in mesangial cells (38). TSP-1 itself is known to promote chemotaxis of leukocytes to inflammatory sites and has a central role in activation and clonal expansion of inflammatory T-cells (39) which would be consistent with the results of our study.

Nevertheless, limitations of this study need to be discussed. First, participation of other activators of TGF-β or direct secretion of the active cytokine by glomerular cells cannot be completely excluded. Second, after single high dose of streptozotocin substantial collateral tissue toxicity may occur, which increases albuminuria (40). TSP-1 mediated effects on destruction/regeneration of beta cells are unlikely, since no differences in blood glucose levels were detected at each time point but cannot completely be excluded. Third, our study can not differentiate, whether effects on diabetic nephropathy are due to a systemic,
bone marrow derived or local lack of TSP-1. Fourth, we show equivalent effects of TSP-1 deficiency in diabetic nephropathy in mice of two different backgrounds but direct comparison of changes during the time course is invalid, since different time points were investigated. Fifth, our STZ-mediated diabetic nephropathy model compared to late diabetic nephropathy in man showed relatively mild lesions with predominant diabetic glomerulopathy limiting the conclusions towards human disease. Nevertheless, the 20 week time point of STZ-induced diabetic nephropathy demonstrated clear cut diabetic glomerular changes that are ameliorated by TSP-1 deficiency. Early treatment or even prophylaxis of diabetic nephropathy in man would be ideal, since TSP-1 expression correlates with disease severity during diabetic nephropathy (16). Furthermore, in man only 30% of all diabetic patients develop diabetic nephropathy suggesting genetic progression factors. In a recent study, peripheral mononuclear blood cells from diabetic patients with and without diabetic nephropathy were screened/compared for candidate target genes using microarray technology. TSP-1 was one out of four differentially regulated genes, suggesting that TSP-1 expression is important for the pathogenesis of diabetic nephropathy in man (41). In addition, the fact that we could show similar results regarding TSP-1 mediated TGF-β activation and matrix accumulation using mice of two different backgrounds strongly supports the relevance of these findings.

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

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References

Figure Legends:

**Figure 1:** Shown is the schematic outline of the experimental design.

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

**Figure 3:** TSP-1 deficiency prevents TGF-β activation in diabetic nephropathy. Active TGF-β (A; B, brown cytoplasmatic staining), the phosphorylation of the TGF-β signaltransduction molecule smad 2/3 (C, brown nuclear staining; D) as well as the TGF-β target gene plasminogen activator inhibitor 1 (PAI-1) (E; F, brown cytoplasmatic staining) was lower during diabetes in TSP-1 deficient mice as shown by immunohistological staining.

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

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

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

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

**Figure 8:** Kidney function was improved in diabetic mice lacking TSP-1. After diabetes induction kidney function was assessed by analyzing proteinuria (A), albuminuria (B) and serum urea (C).
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Fig. 1
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Fig. 7
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Fig. 8