Kidney International, Vol. 53 (1998), pp. 302–311

Thrombospondin 1 precedes and predicts the development of tubulointerstitial fibrosis in glomerular disease in the rat

Christian Hugo, Stuart J. Shankland, Raimund H. Pichler, William G. Couser, and Richard J. Johnson

Division of Nephrology, Department of Medicine, University of Washington, Seattle, Washington, USA

Thrombospondin 1 precedes and predicts the development of tubulointerstitial fibrosis in glomerular disease in the rat. Tubulointerstitial fibrosis is one of the most important histologic features that predicts progression in kidney disease. Thrombospondin 1 is an extracellular matrix protein that can activate latent TGF-β, a cytokine implicated in the pathogenesis of tubulointerstitial fibrosis. We examined the expression of thrombospondin 1 in several animal models of glomerulonephritis (anti-Thy1 model, aminonucleoside nephrosis, passive Heymann nephritis) that are associated with tubulointerstitial disease. Thrombospondin 1 mRNA and protein were transiently increased in tubular cells, myofibroblasts and some macrophages in areas of tubulointerstitial injury. Thrombospondin 1 expression always preceded the development of tubulointerstitial fibrosis, and correlated quantitatively and spatially with the later development of interstitial fibrosis. Thrombospondin 1 expression predicted the severity of tubulointerstitial fibrosis better than the degree of macrophage or myofibroblast accumulation. Thrombospondin 1 expression was associated with increased expression and activation of TGF-β1 and decreased expression of LAP-TGF-β in areas of tubulointerstitial injury. We conclude that thrombospondin 1 is an early marker predicting the development of tubulointerstitial kidney disease. *De novo* expression of thrombospondin 1 is associated and colocalized with increased expression of TGF-β1 and decreased expression of LAP-TGF-β during the development of tubulointerstitial disease *in vivo*. These data are consistent with the possibility that thrombospondin 1 may be an endogenous activator of TGF-β.

Tubulointerstitial inflammation and fibrosis are common in most human glomerular diseases and have been found to predict the risk for progression [1–4]. However, the pathogenic mechanisms causing the interstitial injury are poorly understood. Recent studies have implicated infiltrating macrophages [5, 6], myofibroblasts [7–9], and cytokines such as platelet-derived growth factor BB (PDGF-BB) [10, 11] and transforming growth factor-beta (TGF-β) [12] with the development of tubulointerstitial fibrosis.

Thrombospondin 1 (TSP1) is an extracellular, PDGF and basic fibroblast growth factor (bFGF) inducible protein that has been implicated in fibroblast, smooth muscle cell, and mesangial cell (MC) proliferation and migration [13–15]. In addition, TSP1 has also been shown to activate transforming TGF-β from its latent form by a non-proteolytic mechanism [16, 17]. TGF-β has been linked to matrix expansion in both glomerular and tubulointerstitial disease [12, 18], but the mechanism of TGF-β activation *in vivo* in renal disease remains unknown.

We therefore decided to examine the expression of TSP1 in several experimental models of glomerular disease in which tubulointerstitial injury occurs, to determine if the expression correlated with the development of fibrosis.

Key words: thrombospondin 1, TGF-β activation, tubulointerstitial fibrosis, glomerulonephritis.

Received for publication June 18, 1997
and in revised form September 16, 1997
Accepted for publication September 24, 1997

METHODS

Experimental design

Three models of acute glomerulonephritis were selected in which tubulointerstitial injury and fibrosis develops: (1) anti-Thy1 model, a model of mesangial proliferative nephritis induced by an antibody to the Thy1 antigen on MC; (2) aminonucleoside nephrosis (PAN), a model of minimal change disease induced by a single i.v. injection of puromycin; and (3) passive Heyman nephritis (PHN), a model of membranous nephropathy induced by a single i.p. injection of sheep anti-Fx1A antibody.

At various time points (from 2 days to 21 days) after disease induction renal biopsies (N = 6 to 9 per model and time point) were obtained, immunostained and graded for interstitial TSP1 expression, myofibroblast formation (α-smooth muscle actin), macrophage accumulation (ED-1) and proliferation (PCNA-proliferating cell nuclear antigen). The specific cell types expressing TSP1 in normal and diseased states were identified by double labeling of TSP1 with cell specific markers for myofibroblasts (α-smooth muscle actin), and macrophages (ED-1). To determine if sites of TSP1 expression co-localize with cytokines such as PDGF and TGF-β1 double labeling of TSP1 with antibodies to PDGF-BB, TGF-β1 and the latency associated peptide of TGF-β (LAP-TGF-β1) was performed. The tubulointerstitial expression of TSP1 mRNA was studied by *in situ* hybridization using a specific ^{35}S-labeled cRNA probe. Immunostaining for α-smooth muscle actin was combined with *in situ* hybridization for TSP1 mRNA to identify the cell types expressing TSP1 mRNA.

To determine whether TSP1 expression is associated with increased expression and possibly activation of TGF-β1 the bioactivity of TGF-β1 in cortical tissue samples obtained at day 11 was correlated with the degree of tubulointerstitial TSP1 immunostaining in biopsies from individual control, PAN and PHN animals obtained at the same time (N = 4 to 5). Additionally, double staining for TSP1 and total TGF-β1 in PAN and PHN

animals at day 11 was compared to double staining for TSP1 and latent TGF-β1 on serial sections.

In vivo disease models

Experimental mesangial proliferative nephritis was induced in 180 to 220 g male Wistar rats (Simonsen Laboratories, Gilroy, CA, USA) by i.v. injection of goat anti-rat thymocyte plasma (0.4 cc/100 g body wt) as previously described [19]. Rats were sacrificed and renal biopsies obtained at days 2, 5, 7, 10, 14, and 21 (N = 6 to 9 per timepoint). Six normal rats (day 0) served as controls. Experimental membranous nephropathy (PHN) [20] was induced in male Sprague-Dawley rats (Simonsen) weighing 190 to 210 g by i.p. injection (5 ml/kg body wt) of sheep antibody to Fx1A prepared as previously described [21]. Rats with PHN were sacrificed at 5, 10, 15, and 21 days for renal biopsies (N = 6 at each time point). Aminonucleoside nephrosis (PAN) was induced in 190 to 220 g male Sprague-Dawley rats (Simonsen, Gilroy, CA) by a single i.v. injection of puromycin as described elsewhere [22]. Rats were sacrificed at days 3, 5, 11, 14, and 21 (N = 6 at each time point). In a separate study, rats with PAN and PHN (N = 5 each) underwent survival biopsies [23] at day 11 and were sacrificed at day 21.

Renal morphology and immunohistochemistry

Renal biopsies were fixed in methyl Carnoy's solution and embedded in paraffin. Indirect immunoperoxidase staining of 4 μm sections was performed as described previously [24] using the following primary antibodies: the murine monoclonal antibody TSP A6.1 (IgG_1; kindly provided by Dr. V. Dixit; [25]), a murine monoclonal IgM antibody against PCNA (19A2, Coulter Immunology, Hialeah, FL, USA), a murine monoclonal IgG_1 antibody, ED-1, to a cytoplasmic antigen present in monocytes, macrophages and dendritic cells (Bioproducts for Science, Inc., Indianapolis, IN, USA), a murine monoclonal IgG_2 antibody against α-smooth muscle actin (Sigma Chemical Co., St. Louis, MO, USA); a murine monoclonal IgG antibody, PGF-007, to a 25-amino acid peptide of the human PDGF-B chain (Mochida Pharmaceutical, Tokyo, Japan); a peptide affinity-purified rabbit polyclonal antibody to TGF-β1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA); an affinity-purified polyclonal goat antibody to recombinant human TGF-β1 latency associated peptide (rhLAP TGF-β1; R&D Systems, Minneapolis, MN, USA; [26]), a rabbit polyclonal antibody against rat fibronectin (Chemicon, Temecula, CA, USA), and a rabbit polyclonal antibody against collagen IV (Collaborative Research Inc., Bradford, MA, USA). Controls included omitting the primary antibody and substitution of the primary antibody with pre-immune rabbit or mouse serum. The specificity of the monoclonal anti-TSP1 antibody, TSP A6.1, in rat kidney has been demonstrated elsewhere [14].

For each biopsy 12 to 25 cortical fields measuring 0.25 mm^2 at a $\times$200 magnification using a grid in the eyepiece of the microscope were evaluated in a blinded fashion. Two different methods were used to quantitate immunostaining in the tubulointerstitium. First, TSP1 expression was graded by counting the percentage of positive tubules or tubules that were surrounded by interstitial extra- or intracellular TSP1 staining. Second, a semiquantitative scoring system (0 to 4) was developed: score 0 = 0 to 5%, score 1 = 6 to 25%, score 2 = 26 to 50%, score 3 = 51 to 75%, score 4 = 76 to 100% of tubules are positive or surrounded by positive TSP1 staining within the grid. This semiquantitative scoring system was compared to the above described method to count the percentage of positive tubules during the complete timecourse of PHN. The data from both methods were highly correlated (r = 0.99, $P < 0.001$), which allowed us to evaluate the degree of fibronectin, collagen IV, myofibroblast (α-smooth muscle actin staining) or macrophage accumulation (ED-1 staining) in all models using an equivalent scoring system [0–4]. Interstitial fibrosis was scored semiquantitatively on biopsies stained with Masson's Trichrome using the following scoring system (0 to 3): score 0 = normal interstitium and tubules, score 1 = mild fibrosis with minimal interstitial thickening between the tubules, score 2 = moderate fibrosis with moderate interstitial thickening between the tubules, and score 3 = severe fibrosis with severe interstitial thickening between the tubules.

Immunohistochemical double staining

To determine the cell types expressing TSP1, double immunostaining with the TSP A6.1 antibody and cell specific markers for interstitial cells [macrophages, ED-1 (a murine monoclonal IgG_1); myofibroblasts, α-smooth muscle actin (a murine monoclonal IgG_{2A}); and proliferating cells, PCNA (a murine monoclonal IgM)] was performed using an indirect immunoperoxidase technique. Alpha-smooth muscle actin, ED-1 or PCNA antibody was incubated overnight at 4°C, followed sequentially by biotinylated rabbit anti-mouse IgG_2A, IgG_1 or IgM serum (Zymed, San Francisco, CA, USA), peroxidase conjugated Avidin D (Vector, Burlingame, CA, USA), color development with DAB without nickel chloride. Incubation in 3% H_2O_2/methanol for 20 minutes prevented any remaining peroxidase activity. Subsequently, the TSP A6.1 antibody was applied overnight at 4°C, followed by peroxidase conjugated rat anti mouse IgG_1 antibody and one of the following color reagents by choice: True blue (Kirgaard & Perry Laboratories, Gaithersburg, MD, USA), Vector purple (Vector), DAB with nickel. For sequential double staining with two murine monoclonal IgG_1 antibodies (TSP A6.1 and ED-1) an additional blocking step (after the H_2O_2 treatment) with Fab anti-rabbit and anti-mouse IgG was performed for one hour. Controls included omitting either the primary antibodies or replacing them by mouse serum, as well as omitting either the secondary antibodies. The same protocol was used to double stain for TSP1 and the cytokines PDGF-BB, TGF-β1 or LAP-TGF-β. The reproducibility of this staining pattern with antibodies against TGF-β1 and LAP-TGF-β was also confirmed in Bouin's fixative.

In situ hybridization for thrombospondin 1 mRNA

Thrombospondin 1 mRNA was detected by *in situ* hybridization on formalin-fixed tissue using ^{35}S-labeled anti-sense probes to rat TSP1 [14]. Sense RNA probes were used as controls. *In situ* hybridization for TSP1 mRNA has been shown to correlate tightly with the quantification of TSP1 mRNA by Northern blot analysis of glomerular RNA in the anti-Thy1 model [14]. Hybridization was performed using a protocol of Wilcox et al [27].

Combined immunohistochemistry and *in situ* hybridization

Immunoperoxidase staining for α-smooth muscle actin in combination with *in situ* hybridization for TSP1 mRNA was performed as described elsewhere [14]. In brief, the immunostaining was done first under RNAse free conditions, followed directly by the *in situ* hybridization procedure.

Table 1. Tubulointerstitial fibrosis (Masson's Trichrome), thrombospondin 1 expression, "myofibroblast" (α-sm actin), and macrophage (ED-1) accumulation in anti-Thy 1 nephritis, aminonucleoside nephrosis (PAN), and passive Heymann nephritis (PHN) by immunostaining using a semiquantitative visual scoring system or counting of the percentage of positive tubules per cross section

Parameter	Model	Day 0	Day 2/3	Day 5	Day 7	Day 10/11	Day 14/15	Day 21
Fibrosis	Anti-Thy 1	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.1 ± 0.1	0.3 ± 0.1	0.5 ± 0.4
Trichrome,	PAN	0 ± 0	0 ± 0	0 ± 0	ND	0.1 ± 0.1	0.4 ± 0.1	0.9 ± 0.3
score 0–3	PHN	0 ± 0	ND	0 ± 0	ND	0.4 ± 0.1	0.4 ± 0.4	2.0 ± 0.7
TSP1	Anti-Thy 1	0 ± 0	1.4 ± 0.4	3.6 ± 0.7	2.8 ± 0.8	1.0 ± 0.3	1.0 ± 0.4	0 ± 0
% tubules	PAN	0 ± 0	ND	1.1 ± 1.1	ND	1.8 ± 1.0	7.0 ± 2.6	2.2 ± 0.3
positive	PHN	0 ± 0	ND	5.6 ± 2.7	ND	26.3 ± 2.2	8.8 ± 6.4	3.6 ± 0.1
Myofibroblasts	Anti-Thy 1	0 ± 0	0.6 ± 0.1	0.8 ± 0.2	0.9 ± 0.2	0.9 ± 0.3	1.0 ± 0.3	0.7 ± 0.1
α-sm-actin,	PAN	0 ± 0	ND	0.5 ± 0.2	ND	2.7 ± 0.1	1.8 ± 0.3	1.1 ± 0.1
score 0–4	PHN	0 ± 0	ND	0.9 ± 0.1	ND	2.6 ± 0.3	1.7 ± 0.7	1.5 ± 0.4
Macrophages	Anti-Thy 1	0 ± 0	ND	0.6 ± 0.1	0.8 ± 0.2	0.9 ± 0.3	0.8 ± 0.3	0.4 ± 0.1
ED-1,	PAN	0 ± 0	ND	0.9 ± 0.1	ND	2.2 ± 0.2	2.7 ± 0.4	1.3 ± 0.2
score 0–4	PHN	0 ± 0	ND	0.1 ± 0.1	ND	0.8 ± 0.3	1.2 ± 0.7	1.8 ± 0.4

All the data at the peak in regard to fibrosis, TSP1 expression, myofibroblast-, and macrophage accumulation are significant ($P < 0.01$). Data are mean SD. Abbreviations are: ND, not done; α-sm actin, α smooth muscle actin.

Transforming growth factor-β1 bioactivity

Protein from kidney cortex was extracted using a buffer containing 1% triton, 10% glycerol, 20 mM HEPES, 100 mM NaCl with 10 μg/ml leupeptin, 10 μg/ml antipain, 10 μg/ml pepstatin, 0.1 mM sodium orthovanadate and 50 mM sodium fluoride (reagents purchased from Sigma) as described before [28]. The cortical tissue was homogenized using a polytron for 30 seconds, and placed on ice for 10 minutes, followed by centrifugation at 14000 rpm for five minutes. The protein concentration in the supernatant was measured by BCA protein assay (Pierce, Rockford, IL, USA). Transforming growth factor-β1 bioactivity was measured by a quantitative solid phase enzyme immunoassay according to the instructions of the manufacturer (Quantikine TGF-β1 kit; R & D Systems, Minneapolis, MN, USA). To determine the amount of total versus active TGF-β1 from control ($N = 4$), day 11 PAN ($N = 4$), and day 11 PHN animals ($N = 5$), the protein extracted from each kidney was divided as follows: (*1*) 10 to 15 mg was used to measure the amount of active TGF-β1 present (no acid activation); (*2*) 1 mg of protein was acid activated with 1 N HCl to determine the total amount of TGF-β1 that can be activated. The glomerular protein extracts were added to a microtiter plate coated with the TGF-β receptor that only allows binding of active TGF-β, and were incubated for three hours. After washing, a horseradish peroxidase-linked polyclonal antibody specific for TGF-β1 was added for two hours, followed by a substrate solution. The optical density was measured by 450 nm. The concentration of TGF-β1 (pg/μg protein) from the cortical preparations was measured by comparing the optical density of each sample to a standard curve prepared using recombinant TGF-β1.

Statistical analysis

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

RESULTS

Interstitial fibrosis occurs late and to different degrees in all three models of glomerular disease

As described previously [23], all three models of glomerular disease (anti-Thy1, PAN, PHN) were accompanied by tubulointerstitial injury with tubular dilation and atrophy, tubular cast formation, infiltration of mononuclear cells and accumulation of extracellular matrix. Using Masson's Trichrome staining technique, which identifies matrix proteins (mainly collagens), areas of interstitial fibrosis were easily detectable and semiquantitatively scored from 0 to 3 (Table 1) as described in the **Methods** section. While the onset of (detectable) interstitial fibrosis in all models started uniformly around days 10 to 14 after disease induction and increased progressively during disease, the degree of interstitial fibrosis in these models varied considerably. Interstitial fibrosis was mildest in the anti-Thy1 model, moderate in PAN, but dramatic in PHN (Table 1).

Thrombospondin 1 protein and mRNA are up-regulated in the tubulointerstitium in all models of glomerular disease and precede tubulointerstitial fibrosis

In the normal rat renal cortex, TSP1 protein (Fig. 1A) and mRNA (Fig. 1 B, C) were rarely detectable in parietal epithelial cells of Bowman's capsule and juxtaglomerular cells, but absent within the tubulointerstitium. In contrast to the cortical tubulointerstitium, TSP1 protein and mRNA were expressed by tubular and collecting duct cells within the medulla (not shown).

In all three models of GN, TSP1 protein and mRNA were expressed *de novo* in the cortical tubulointerstitium early in the course of disease (Table 1 and Fig. 1). In the anti-Thy1 model, only a mild and focal increase of TSP1 protein was observed, starting very early on day 2, peaking on day 5 parallel to the glomerular expression [14] and subsiding thereafter. Despite the early onset of TSP1 expression, interstitial fibrosis was not detected before day 14 in the anti-Thy1 model, when TSP1 expression was already subsiding. In PAN, TSP1 was also only focally increased, starting early on day 5 and peaking on day 14.

Detectable interstitial fibrosis started on day 14 and peaked on day 21. Thrombospondin 1 was also induced in the tubulointerstitium of the PHN model, where its expression started on day 5, peaked on day 10 and declined later in disease. Again, interstitial fibrosis developed after the onset of TSP1 expression, being minimally elevated at the peak of TSP 1 expression on day 10, but substantially elevated later on in disease (Table 1).

Thrombospondin 1 protein expression was paralleled by tubulointerstitial TSP1 mRNA expression in all three models (as assessed by *in situ* hybridization; Fig. 1) and followed a specific pattern. The early expression of TSP1 (day 5) was commonly confined to proximal and distal tubular cells after injury (Fig. 1D, E, F). At later time points (day 10), TSP1 was still predominantly expressed by tubular cells, but was also present in the extracellular space in the interstitium and in a heterogenous cell population of round and spindle shaped interstitial cells (Fig. 1 G, H, I).

Identification of the cells expressing thrombospondin 1 protein and mRNA

Most of the TSP1 protein and TSP1 mRNA is produced by injured/dilated proximal and distal tubular cells in the typical sequence during all three disease models as described above. In order to characterize the fewer cells expressing TSP1 in the interstitium, we performed double immunolabeling for TSP1 (TSP A6.1) and proliferating cells (PCNA), macrophages (ED-1), or activated interstitial (myo-) fibroblasts (α-smooth muscle actin). Double immunostaining of TSP A6.1 with PCNA, ED-1 or α-smooth muscle actin antibody in these disease models showed that, although some TSP1 expressing interstitial cells were also proliferating and/or ED-1 positive macrophages, the majority of TSP1 positive interstitial cells were α-smooth muscle actin positive myofibroblasts (Fig. 2A; PHN day 10). This cellular expression pattern was confirmed by combining *in situ* hybridization of TSP1 mRNA with immunostaining for α-smooth muscle actin, which showed localization of TSP1 mRNA in α-smooth muscle actin positive myofibroblasts (Fig. 2B; PHN day 10) besides the predominant tubular expression.

Thrombospondin 1 expression differs from that of other extracellular matrix proteins

To determine whether the time course of TSP1 expression is similar to or different from other extracellular matrix proteins, the kinetics of collagen IV and fibronectin were compared to TSP1 in all models. While tubulointerstitial TSP1 was induced early and only transiently in these disease models, interstitial staining for collagen IV and fibronectin increased later and paralleled closely the timecourse of interstitial fibrosis as assessed by the Trichrome staining. Figure 3 shows the typical time course of TSP1 compared to collagen IV and fibronectin expression in the PHN model.

Interstitial macrophages and "myofibroblasts" predict interstitial fibrosis less well than thrombospondin 1 expression

In all models examined the development of tubulointerstitial fibrosis was preceded by the appearance of ED-1 positive macrophages (Table 1) and α-smooth muscle actin positive myofibroblasts (Table 1) within the tubulointerstitium, similar to the increase in TSP1 expression. However, while the degree of early TSP1 expression correlated closely with the extent of interstitial fibrosis later in all models, neither the degree of early macrophage nor of early myofibroblast accumulation in the interstitium correlated with the severity of interstitial fibrosis in these glomerulonephritis models (Table 2). While the number of infiltrating macrophages in the early phase was greatest in PAN, interstitial fibrosis and TSP1 expression in this model was only moderate. In the PHN model, which is characterized by more severe interstitial fibrosis and TSP1 expression, the interstitial infiltrate of macrophages was less than in the PAN model for all time points except day 21. Similarly, the degree of myofibroblast accumulation did not differ in the PAN or PHN model despite significant differences in the degree of interstitial fibrosis. Only on day 21, when tubulointerstitial fibrosis was already established, macrophage and myofibroblast accumulation correlated well with the degree of fibrosis (Table 2).

In contrast, the peak TSP1 expression occurring early in disease correlated closely with the maximal interstitial fibrosis occurring at day 21 in these disease models (anti-Thy1 model $<$ PAN $<$ PHN; Table 1). To examine this directly, expression of TSP1 on day 11 in PHN and PAN animals (survival biopsies) was correlated with the degree of interstitial fibrosis on day 21 in the same animals (sacrificial biopsies). Figure 4 demonstrates that the TSP1 expression on day 11 in these individual animals correlated tightly with the degree of interstitial fibrosis on day 21 ($r = 0.95$, $P < 0.0001$). In addition, animals that showed a more pronounced TSP1 expression in areas of the juxtamedullary tubulointerstitium on day 11 also demonstrated a predominant juxtamedullary pattern of interstitial fibrosis on day 21. Animals with diffuse tubulointerstitial TSP1 expression on day 11 showed diffuse distribution of fibrosis later.

Thrombospondin 1 expression colocalizes with TGF-β1 in tubulointerstitial disease, but not with PDGF-BB expression

Interstitial PDGF-BB expression in all disease models examined did not show major changes compared to normal controls. Tubular PDGF-BB expression was focally increased during the time course of these glomerulonephritis models. However, double labeling of TSP1 with PDGF-BB did not show coexpression (not shown).

However, both TSP1 and TGF-β1 were increased and coexpressed in areas of tubulointerstitial injury. Transforming growth factor-β1 in the normal kidney is hardly expressed in glomerulus (possibly at low levels in glomerular epithelial cells), but is abundant in proximal and distal tubular cells. This expression pattern could also be found by staining with an antibody against LAP-TGF-β, suggesting that most of the TGF-β1 is in its latent form. In disease, while TSP1 and total TGF-β1 were increased in tubular and interstitial cells in areas of tubulointerstitial injury, LAP-TGF-β was predominantly decreased in these areas. We performed double staining for TSP1 and TGF-β1 (Fig. 5A) as well as for TSP1 and LAP-TGF-β1 (Fig. 5B) on serial sections. Comparing these consecutive sections, we could identify areas of injury in which increased TSP1 expression was associated with increased total TGF-β1 expression (Fig. 5A), but not with a comparable increase or frequently even a decrease in LAP-TGF-β (Fig. 5B), suggesting a net increase in the active fraction of TGF-β1 (without LAP) in these areas of *de novo* expression of TSP1. Interestingly, the glomerular expression of TGF-β also increased concomittently with TSP1 in experimental mesangial proliferative nephritis (anti-Thy 1 model). In contrast, no increase

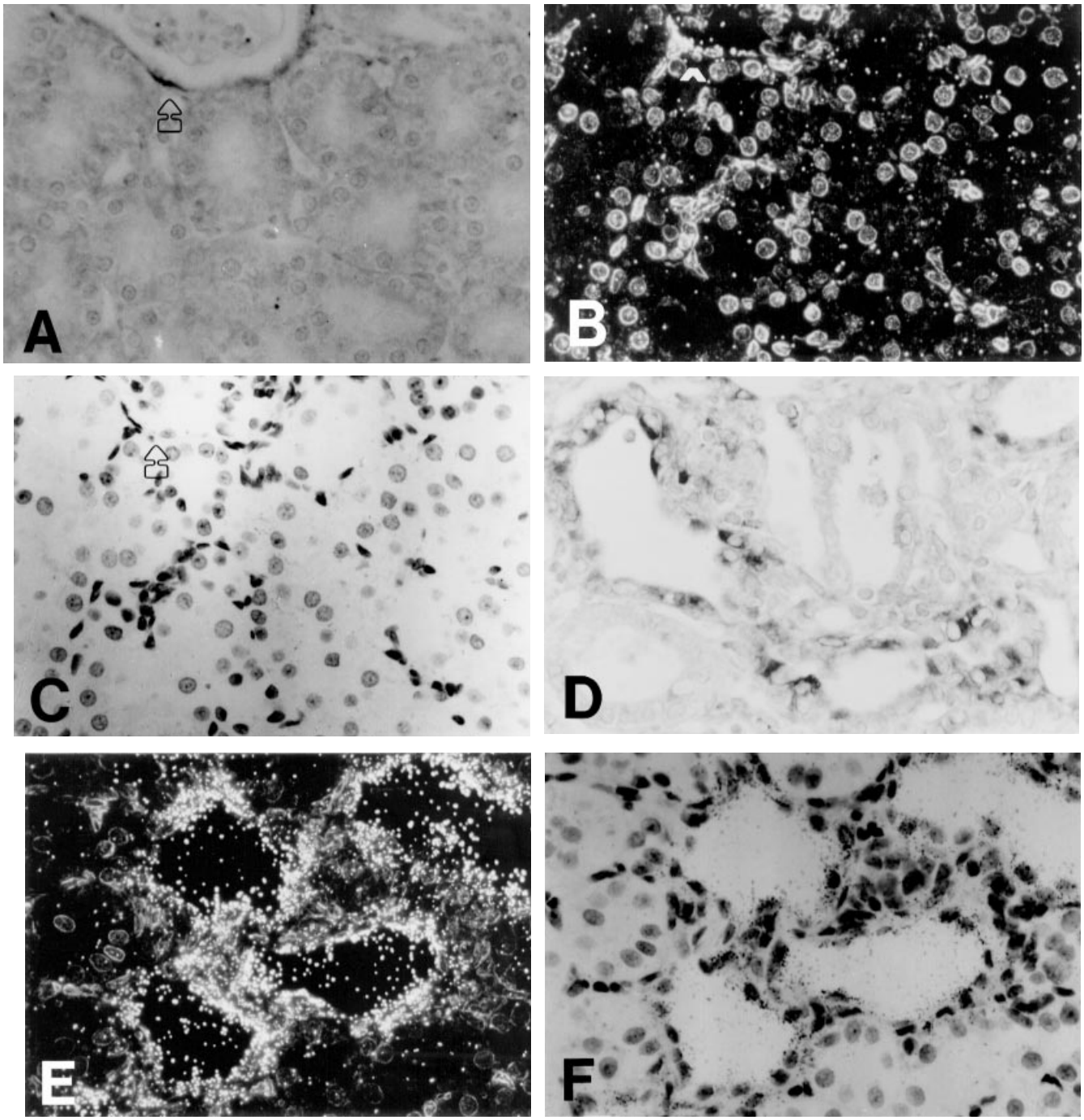
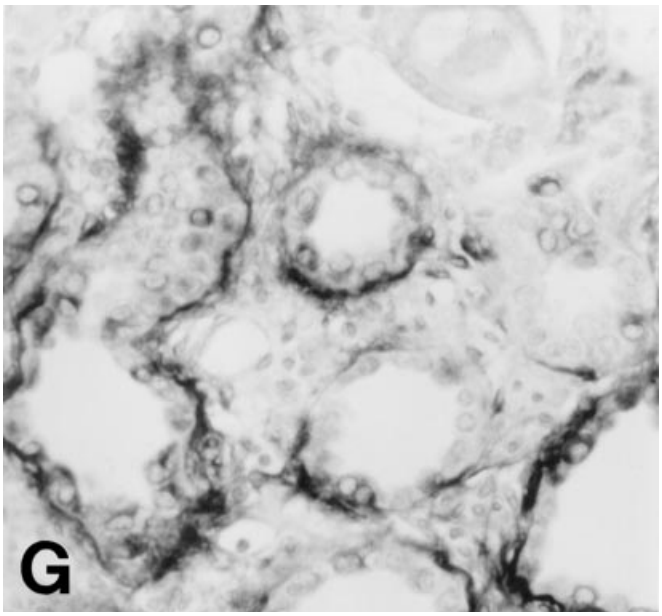


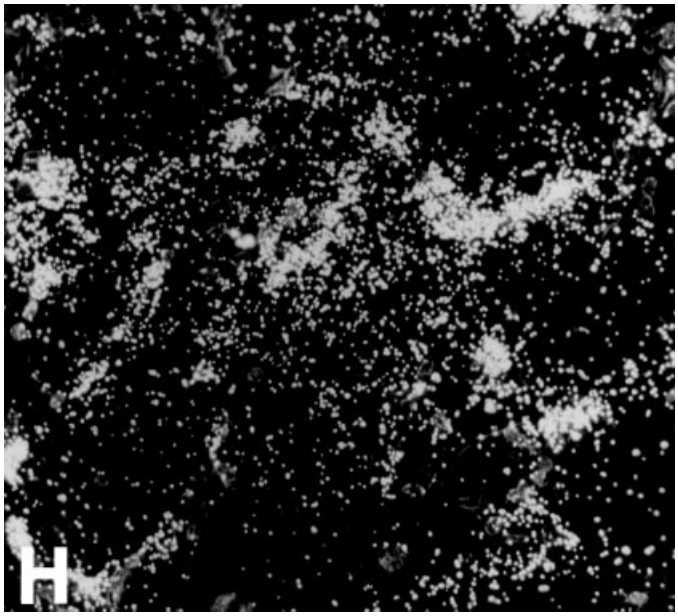
Fig. 1. Thrombospondin 1 (TSP1) protein and mRNA are expressed *de novo* in areas of tubulointerstitial injury accompanying glomerular disease. In normal rats, TSP1 protein (dark immunostaining, *A*) and mRNA (*in situ* hybridization, *B*) darkfield showing positive signal by white grains, (*C*) brightfield showing positive signal by dark grains] were expressed occasionally by parietal glomerular epithelial cells (arrow), but were absent in the tubulointerstitium. The early expression of TSP1 protein (*D*) and mRNA [(*E*) darkfield, (*F*) brightfield] was commonly confined to proximal and distal tubular cells after injury and was mainly intracellular in location (here PHN day 5).

of either TSP1 or TGF-β1 were noted in glomeruli in the PHN and PAN models, consistent with prior studies [28].

Interstitial thrombospondin 1 expression is associated with TGF-β1 expression and bioactivity in PAN and PHN

Using an enzyme immunoassay, we determined the amount of active and total TGF-β1 in cortical tissue samples from rats with PAN and PHN at day 11. Since immunostaining for TGF-β1 did not show any significant increases in TGF-β1 within the glomerulus at this time point, differences in TGF-β1 bioactivity from cortical samples of diseased animals were considered to reflect changes in the tubulointerstitium. The measurement of the active fraction of TGF-β1 (without acid activation, 0.5 ± 0.1 pg TGF-β1/mg protein) in normal cortical tissue showed that only about





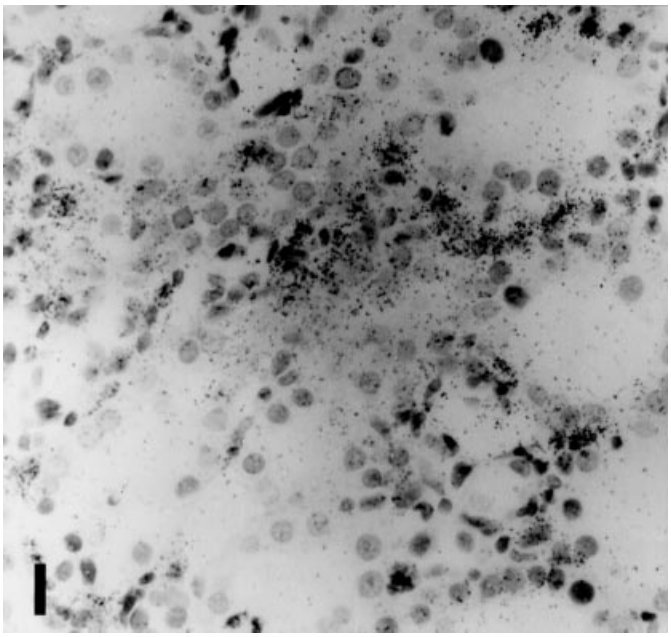


Fig. 1. *Cont'd.* At later time points, TSP1 protein (*G*) and mRNA [(*H*) darkfield, (*I*) brightfield] were identified in the extracellular space in the interstitium and also were present in a heterogenous cell population of round and spindle shaped interstitial cells (here PHN day 10).

1% of total TGF-β1 (after acid activation, 50.0 ± 2.5 pg TGF-β1/mg protein) is active. This result is consistent with a widely overlapping distribution of TGF-β1 and LAP-TGF-β (see immunostaining above). In diseased animals at day 11, the active TGF-β1 fraction (no acid activation) increased significantly ($P < 0.05$) up to three times (PAN day 11, 1.5 ± 0.3 pg TGF-β1/mg protein; PHN day 11, 1.5 ± 0.5 pg TGF-β1/mg protein) versus normal controls. The total TGF-β1 bioactivity (after acid activation) also increased significantly ($P < 0.05$) up to two times in diseased animals versus normal controls (PAN day 11, 101.9 ± 21.1 pg TGF-β1/mg protein; PHN day 11, 89.0 ± 36.3 pg TGF-β1/mg protein). Comparison of the degree of tubulointerstitial TSP1 expression with the amount of TGF-β1 bioactivity (both active and total) in individual animals at day 11 in PAN, PHN, or controls revealed a high correlation ($r = 0.93/0.94$, $P < 0.001$; Fig. 6 A, B).

DISCUSSION

Tubulointerstitial fibrosis is a major determinant for both renal function and prognosis in glomerular disease [1–4, 29]. Thrombospondin 1 is an extracellular matrix protein that has been shown to have a role in PDGF- and bFGF-mediated proliferation in various cell types, and to also be an endogenous activator of TGF-β [14–16]. To study the role of TSP1 in the development of tubulointerstitial fibrosis, we compared several models of glomerulonephritis (anti-Thy1 model, PAN, and PHN) characterized by

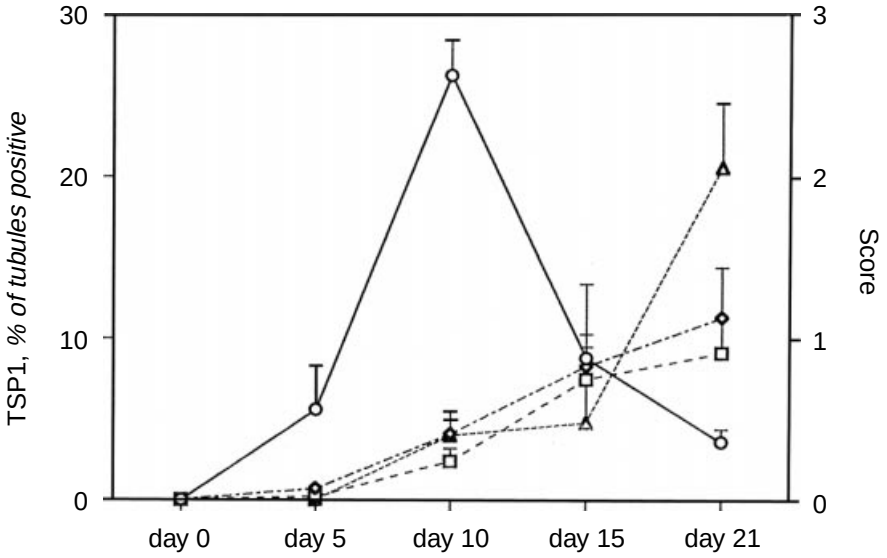


Fig. 3. Thrombospondin (TSP1) expression precedes the accumulation of other extracellular matrix proteins. This graph illustrates the kinetics of tubulointerstitial collagen IV (◇) and fibronectin (□) expression in comparison to TSP1 (○) in the PHN model (similar in other models examined). While tubulointerstitial TSP1 was induced early and only transiently in PHN, collagen IV and fibronectin were increased later and paralleled closely the time course of interstitial fibrosis (△; see Table 1).

Table 2. Regression analysis (*r*-value) of TSP 1 expression, myofibroblast (α-sm actin), or macrophage (ED-1) accumulation on days 10/11, 14/15 or 21 in anti-Thy 1 disease, PAN, and PHN for the prediction of tubulointerstitial fibrosis (Trichrome) on day 21

Analysis	Day 10/11	Day 14/15	Day 21
TSP1/fibrosis	0.98	0.83	0.92
Myofibroblast/ fibrosis	0.65	0.55	0.96
Macrophages/ fibrosis	0.34	0.1	0.89

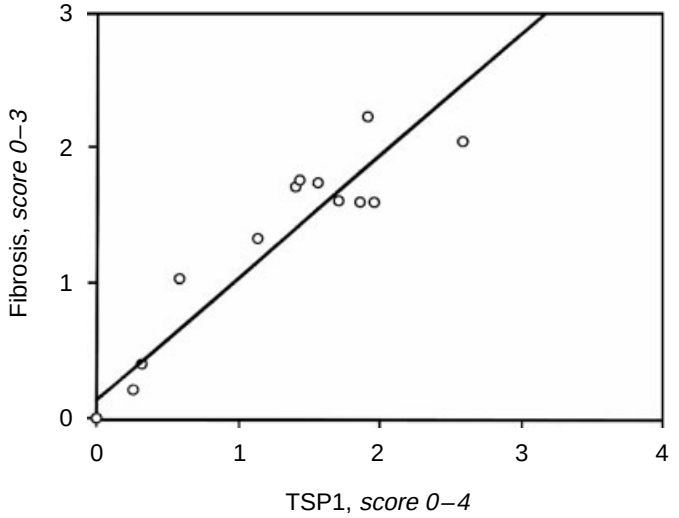


Fig. 4. Tubulointerstitial thrombospondin (TSP1) expression predicts the development of interstitial fibrosis. Expression of TSP1 (immunostaining, score 0 to 4) on day 11 in control, PHN and PAN animals (survival biopsies) was correlated with the degree of interstitial fibrosis (Trichrome staining, score 0 to 3) on day 21 in the same animals (sacrifical biopsies). TSP1 expression correlated tightly with the degree of interstitial fibrosis ($r = 0.95$, $P < 0.0001$) in these individual animals.

tubulointerstitial disease with tubular atrophy and dilation, mononuclear cell infiltrate, myofibroblast formation, and matrix expansion. Thrombospondin 1 mRNA and protein expression were transiently expressed (*de novo*) in areas of tubulointerstitial injury; TSP1 expression localized preferentially to tubular cells, and to a lesser degree to inflammatory interstitial myofibroblasts and macrophages, and correlated quantitatively and spatially with the later development of tubulointerstitial fibrosis.

In all disease models studied, TSP1 mRNA and protein were *de novo* and transiently expressed in areas of tubulointerstitial injury, starting as early as day 2 in anti-Thy1 disease and day 5 in PAN, and PHN, peaking between day 5 (anti-Thy1 disease) and day 10/11 (PHN/PAN), and subsiding afterwards. Thrombospondin 1 (mRNA and protein) expression was initially intracellular and limited (mainly) to tubular cells and later was present in extracellular areas in the interstitium. Later in disease TSP1 expression was also localized to a lesser degree to myofibroblasts and macrophages (shown by double staining for TSP1 and cell specific markers), but still seems to be predominantly produced by injured tubular cells. The early and transient tubulointerstitial expression of TSP1 is unique and different from other common extracellular matrix proteins such as fibronectin and collagen IV. These latter extracellular proteins paralleled the development of interstitial fibrosis suggesting that they are more permanently incorporated in the interstitial matrix and participate structurally in the process of interstitial fibrosis, in contrast to TSP1.

A major finding was that early tubulointerstitial TSP1 expression correlated with both the development and degree of tubulointerstitial fibrosis in all three disease models (Table 2). This was further supported by studies in individual animals with PAN or PHN in which both the site and degree of TSP1 expression at day 11 correlated with site and degree of tubulointerstitial fibrosis at day 21. Thrombospondin 1 expression was superior to the degree of early macrophage or myofibroblast accumulation in predicting the later development of fibrosis. These data do not question that macrophages or myofibroblasts are involved in the development of tubulointerstitial fibrosis, as previous studies suggest [7–9, 12, 30–32], but suggest that other factors such as local expression of TSP1 and possibly other cytokines, may have an important influence on the fibrotic process.

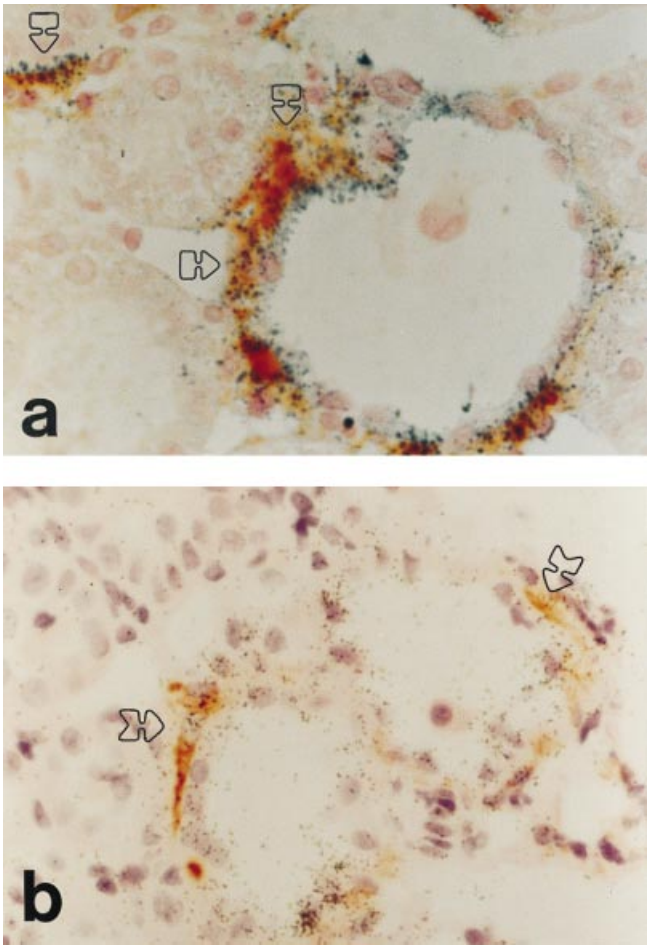


Fig. 2. Alpha-smooth muscle actin positive myofibroblasts are a source of interstitial thrombospondin 1 (TSP1) protein (*a*) and mRNA (*b*) expression during the development of fibrosis. While TSP1 is predominantly expressed in tubular cells during TI-injury, double immunostaining for α-smooth muscle actin (brown) and TSP1 (blue grains) identified that some α-smooth muscle actin positive interstitial cells were also positive for TSP1 (arrows in a, here PHN day 10). Combination of *in situ* hybridization for TSP1 mRNA (dark grains) and immunostaining for α-smooth muscle actin (brown) confirmed expression of TSP1 mRNA by interstitial myofibroblasts (arrows in b, here PHN day 10). Tubular expression of TSP1 protein (a, blue grains) and TSP1 mRNA (b, black grains) during disease can also be appreciated.

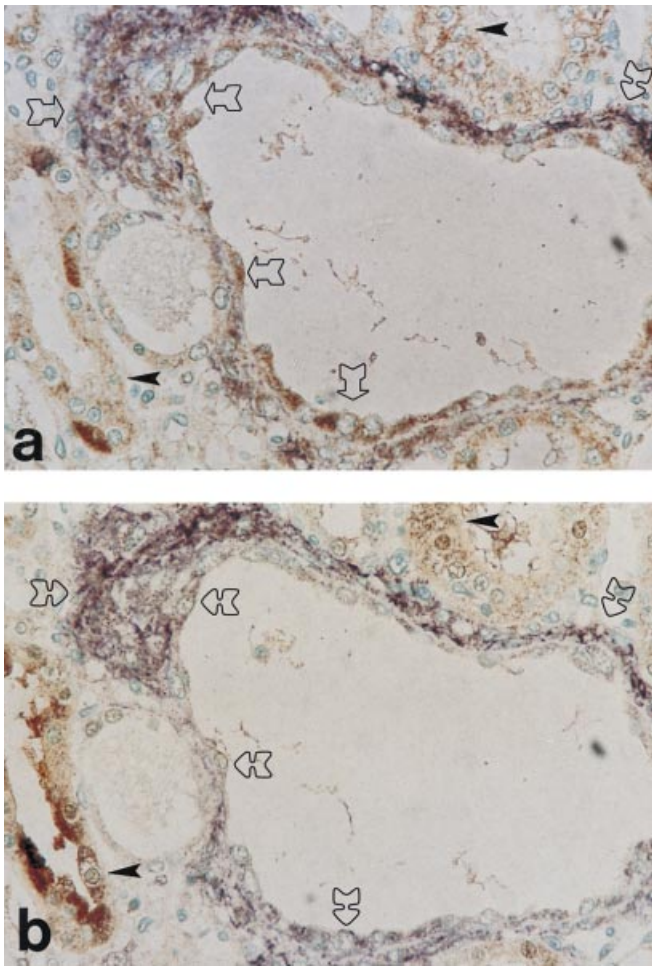


Fig. 5. *De novo* expression of thrombospondin (TSP1) co-localizes to sites of increased transforming growth factor beta1 (TGF-β1) and decreased latency associated peptide of TGF-β (LAP-TGF-β) expression during tubulointerstitial injury. These photographs reveal double immunostaining for TSP1 (blue color in a and b) and TGF-β1 (brown color in a) as well as TSP1 and LAP-TGF-β1 (brown color in b) on serial sections (A/B). In disease (here PAN day 11), while TSP1 TGF-β1 were increased and coexpressed in tubular and interstitial cells in areas of tubulointerstitial injury (arrow in *a*), LAP-TGF-β1 was decreased in the same area of the consecutive section (arrow in *b*). The equal staining intensity of TGF-β1 (a) and LAP-TGF-β1 (b) of TSP1 negative, nondilated (and presumably uninjured) tubules can be noted (arrowheads).

The temporal and spatial relationship of TSP1 expression to the tubulointerstitial fibrosis suggests a functional/causal relationship. Thrombospondin 1 has been implicated in proliferation in several cultured cell types (fibroblasts, mesangial, endothelial, and vascular smooth muscle cells), either via stimulation of PDGF or independently [13, 33, 34]. While the time course of tubulointerstitial proliferation (peaking at day 10 in PHN) was very similar to the transient expression of TSP1, double staining of TSP1 with PCNA (a marker of proliferation) did not show colocalization (not shown). Within the tubulointerstitium, PDGF was only mildly up-regulated in certain tubular areas of injury, but not in interstitial cells and did not colocalize with TSP1 expression.

Thrombospondin 1 has also been shown to stimulate the expression of α-smooth muscle actin by fibroblasts in a model of aortic explants [35] and to mediate monocyte diapedesis *in vitro* [36]. Although macrophages and myofibroblasts are coexpressed with TSP1 in areas of tubulointerstitial injury, the correlation of TSP1 expression with these cell types was not strong, suggesting that TSP1 does not have a major role in the accumulation of these cell types at the sites of injury.

The fibrosis-promoting effects of TGF-β have been well established *in vitro* and *in vivo* in diseases of the kidney and several other organs [37]. Transforming growth factor-β is considered to be a major cytokine linking tissue injury to scarring, not only in the glomerulus [18, 38], but also within the tubulointerstitium, including in the PAN [39] and PHN [40] models. Transforming growth factor-β is secreted in a biologically inactive or latent form that consists of a mature, biologically active component noncovalently associated with the TGF-β latency associated peptide (LAP-TGF-β) and variably with a latent TGF-β binding protein (LTBP)

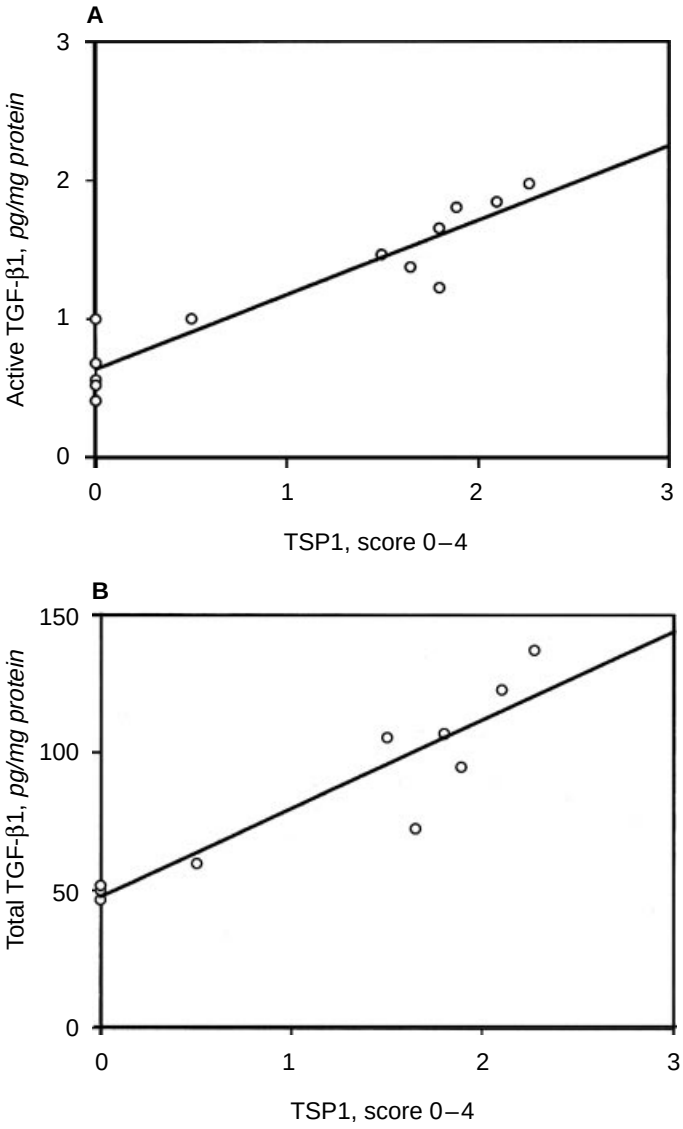


Fig. 6. Interstitial thrombospondin (TSP1) expression is linked to transforming growth factor β1 (TGF-β1) bioactivity measurements in PAN and PHN rats. These graphs demonstrate that TGF-β1 bioactivity measurements [pg/mg protein, (*A*) active TGF-β1 without acid activation, (*B*) total TGF-β1 after acid activation) from cortical samples of control and diseased animals (PAN, PHN day 11) correlated with tubulointerstitial TSP1 expression (immunostaining, score 0 to 4) in the same animals (A, $r = 0.93$, $P < 0.001$; B, $r + 0.94$, $P < 0.001$).

[41–43]. Despite great interest and possible therapeutic relevance, the endogenous factor(s) mediating the activation of this TGF-β complex during *in vivo* disease processes are still unknown.

Recently, TSP1 has been shown to bind and activate TGF-β *in vitro* [16, 17]. Activation of latent TGF-β by TSP1 is mediated by two sequences present in the type 1 repeats of TSP1 [44] a sequence that binds to TGF-β and may orient the TSP1 molecule and a second sequence that activates latent TGF-β [45]. This study does not prove, but is consistent with the hypothesis that TSP1 may be an endogenous activator of latent TGF-β. First, the *de novo* expression of TSP1 frequently colocalized with increased TGF-β1 in areas of tubulointerstitial injury. Second, cortical TGF-β1 bioactivity measurements (total and active) at the peak of TSP1 expression at day 11 in PAN and PHN were also increased up to two- to threefold and highly correlated with the degree of tubulointerstitial TSP1 expression in the same animals (Fig. 6 A and B). It is interesting to note that even in diseased animals the great majority of TGF-β1 is in its latent form, unlike *in vitro* experiments where up to 60% of the latent TGF-β can be activated by TSP1 for example [17]. In this regard it has to be considered that measurements of TGF-β1 activity in whole cortical tissue, where large areas of the tubulointerstitium are unaffected, do not necessarily reflect the focal areas where TGF-β1 is being activated. Third, TSP1 positive areas of tubulointerstitial injury were frequently accompanied by an increase in total TGF-β1, but a decrease in LAP-TGF-β1. The observation of *de novo* expression of TSP1 with expression of TGF-β1 and decreased expression of LAP-TGF-β suggests that TSP1 may be involved in the local activation of TGF-β.

In conclusion, *de novo* expression of TSP1 in the tubulointerstitium of several experimental glomerulonephritis models preceded and highly predicted the development of interstitial fibrosis. Thrombospondin 1 expression was associated with TGF-β1 expression and decreased LAP-TGF-β expression in areas of tubulointerstitial injury. Future studies with specific peptides blocking TGF-β1 activation by TSP1 may prove the attractive hypothesis that TSP1 is an endogenous activator of TGF-β1 and an important mediator of interstitial fibrosis.

ACKNOWLEDGMENTS

The authors are grateful to Dr. V. Dixit (University of Michigan, Ann Arbor, MI) for providing the murine monoclonal anti-thrombospondin antibody A6.1. This work was supported in part by United States Public Health Service grants (DK-43422, DK-02142, DK34198, DK51096, DK52121, and HL 18645) and by an NIH George O'Brien Kidney Center grant (DK47659), and by a post-doctoral stipend of the Deutsche Forschungsgemeinschaft as well as the National Kidney Foundation to C.H. Portions of this work were presented at the American Society of Nephrology annual meeting, San Diego, CA, October 26–29, 1995 and published in abstract form (*J Am Soc Nephrol* 5:782, 1994).

Reprint requests to Christian Hugo, M.D., Division of Nephrology, Box 356 521, BB-1265 Health Sciences, University of Washington Medical Center, Seattle, Washington 98195, USA.

APPENDIX

Abbreviations used in this paper are: bFGF, basic fibroblast growth factor; DAB, diaminobenzidine; GN, glomerulonephritis; LAP, latency associated peptide; MC, mesangial cell; PAN, puromycin aminonucleoside nephrosis; PCNA, proliferating cell nuclear antigen; PHN, Passive Heymann nephritis; PDGF, platelet-derived growth factor; TGF-β, transforming growth factor-β; TSP1, thrombospondin 1.

REFERENCES

1. Schainuck L, Stricker G, Cutler R, Benditt E: Structural-functional correlations in renal disease. Part II. The correlations. *Hum Pathol* 1:631–641, 1970
2. Bohle A, Glomb D, Grund K, Mackensen S: Correlations between relative interstitial volume of the renal cortex and serum creatinine concentration in minimal changes with nephrotic syndrome and in focal sclerosing glomerulonephritis. *Virchows Arch* 376:221–232, 1977
3. Bohle A, Grund K, Mackensen S, Tolon M: Correlations between renal interstitium and level of serum creatinine. Morphometric investigations of biopsies in perimembranous glomerulonephritis. *Virchows Arch* 373:15–22, 1977
4. Mackensen S, Grund K, Sindjic M, Bohle A: Influence of the renal cortical interstitium on the serum creatinine concentration and serum

creatinine clearance in different chronic sclerosing interstitial nephritides. *Nephron* 24:30–34, 1979
5. Nicolic-Paterson D, Lan H, Atkins R: Macrophages in renal injury. *Kidney Int* 45:79–82, 1994
6. Klahr S: Interstitial macrophages. *Semin Nephrol* 13:488–495, 1993
7. Alpers C, Hudkins K, Floege J, Johnson R: Human renal cortical interstitial cells with some features of smooth muscle cells participate in tubulointerstitial and crescentic glomerular injury. *J Am Soc Nephrol* 5:201–210, 1994
8. Alpers C, Pichler R, Johnson R: Phenotypic features of cortical interstitial cells potentially important in fibrosis. *Kidney Int* 49:28–31, 1996
9. Diamond J, van Goor H, Ding G, Engelmyer E: Myofibroblasts in experimental hydronephrosis. *Am J Pathol* 146:121–129, 1995
10. Kliem V, Johnson R, Alpers C, Yoshimura A, Couser W, Koch K, Floege J: Mechanisms involved in the pathogenesis of tubulointerstitial fibrosis in 5/6-nephrectomized rats. *Kidney Int* 49:666–678, 1996
11. Tang WW, Ullich TR, Lacey DL, Hill DC, Qi M, Kaufman SA, Van Gy, Tarpely JE, Yee JS: Platelet-derived growth factor-BB induced renal tubulointerstitial myofibroblast formation and tubulointerstitial nephritis. *Am J Pathol* 148:1169–1180, 1996
12. Eddy A: Experimental insights into the tubulointerstitial disease accompanying primary glomerular lesions. *J Am Soc Nephrol* 5:1273–1287, 1994
13. Frazier W: Thrombospondins. *Curr Opin Cell Biol* 3:792–799, 1991
14. Hugo C, Pichler R, Meek R, Gordon K, Kyriakides T, Floege J, Bornstein P, Couser W, Johnson RJ: Thrombospondin 1 is expressed by proliferating mesangial cells in vivo and is upregulated by PDGF and bFGF. *Kidney Int* 48:1846–1856, 1995
15. Bornstein P: Diversity of function is inherent in matricellular proteins: An appraisal of thrombospondin 1. *J Cell Biol* 130:503–506, 1995
16. Schultz-Cherry S, Murphy-Ullrich JE: Thrombospondin causes activation of latent transforming growth factor-beta secreted by endothelial cells by a novel mechanism. *J Cell Biol* 122:923–932, 1993
17. Schultz-Cherry S, Ribeiro S, Gentry L, Murphy-Ullrich JE: Thrombospondin binds and activates the small and large forms of latent transforming growth factor-beta in a chemically defined system. *J Biol Chem* 269:26775–26782, 1994
18. Border WA, Okuda S, Languino LR, Sporn MB, Ruoslahti E: Suppression of experimental glomerulonephritis by antiserum against transforming growth factor beta 1. *Nature* 346:371–374, 1990
19. Johnson RJ, Pritzl P, Iida H, Alpers CE: Platelet-complement interactions in mesangial proliferative nephritis in the rat. *Am J Pathol* 138:313–321, 1991
20. Alousi M, Post R, Heymann W: Experimental autoimmune nephrosis in rats. *Am J Pathol* 54:47–71, 1969
21. Salant DJ, Darby C, Couser WG: Experimental membranous glomerulonephritis in rats. Quantitative studies of glomerular immune deposit formation in isolated glomeruli and whole animals. *J Clin Invest* 66:71–81, 1980
22. Pichler R, Giachelli CM, Lombardi D, Pippin J, Gordon K, Alpers CE, Schwartz SM, Johnson RJ: Tubulointerstitial disease in glomerulonephritis. Potential role of osteopontin (uropontin). *Am J Pathol* 144:915–926, 1994
23. Floege J, Burns MW, Alpers CE, Yoshimura A, Pritzl P, Gordon K, Seifert RA, Bowen PDF, Couser WG, Johnson RJ: Glomerular cell proliferation and PDGF expression precede glomerulosclerosis in the remnant kidney model. *Kidney Int* 41:297–309, 1992
24. Johnson RJ, Garcia RL, Pritzl P, Alpers CE: Platelets mediate glomerular cell proliferation in immune complex nephritis induced by anti-mesangial cell antibodies in the rat. *Am J Pathol* 136:369–374, 1990
25. Dixit VM, Galvin NJ, O'Rourke KM, Frazier WA: Monoclonal antibodies that recognize calcium-dependent structures of human thrombospondin. Characterization and mapping of their epitopes. *J Biol Chem* 261:1962–1968, 1986
26. Barcellos-Hoff M, Derynck R, Tsang M-S, Weatherbee J: Transforming growth factor-beta activation in irradiated murine mammary gland. *J Clin Invest* 93:892–899, 1994
27. Wilcox JN, Smith KM, Schwartz SM, Gordon D: Localization of tissue factor in the normal vessel wall and in the atherosclerotic plaque. *Proc Nat Acad Sci USA* 86:2839–2843, 1989
28. Shankland SJ, Pippin J, Pichler RH, Gordon K, Friedman S, Gold LI, Johnson RJ, Couser WG: Differential expression of transforming growth factor-β isoforms and receptors in experimental membranous nephropathy. *Kidney Int* 50:116–124, 1996
29. Nath K: Tubulointerstitial changes as a major determinant in the progression of renal damage. *Am J Kidney Dis* 20:1–17, 1992
30. Lan H, Paterson D, Atkins R: Initiation and evolution of interstitial leukocytic infiltration in experimental glomerulonephritis. *Kidney Int* 40:425–433, 1991
31. Diamond J, Pesek-Diamond I: Sublethal X-irradiation during acute puromycin nephrosis prevents late renal injury: Role of macrophages. *Am J Physiol* 260:F779–F786, 1991
32. Harris K, Lefkowith J, Klahr S, Schreiner G: Essential fatty acid deficiency ameliorates acute renal dysfunction in the rat after the administration of the aminonucleoside of puromycin. *J Clin Invest* 86:1115–1123, 1990
33. Majack RA, Goodman LV, Dixit VM: Cell surface thrombospondin is functionally essential for vascular smooth muscle cell proliferation. *J Cell Biol* 106:415–422, 1988
34. Marinides GN, Suchard SJ, Mookerjee BK: Role of thrombospondin in mesangial cell growth: Possible existence of an autocrine feedback growth circuit. *Kidney Int* 46:350–357, 1994
35. Nicosia RF, Tuszynski GP: Matrix-bound thrombospondin promotes angiogenesis in vitro. *J Cell Biol* 124:183–193, 1994
36. Huber A, Ellis S, Johnson K, Dixit V, Varani J: Monocyte diapedesis through an in vitro vessel wall construct: Inhibition with monoclonal antibodies to thrombospondin. *J Leukoc Biol* 52:524–528, 1992
37. Border WA, Brees D, Noble NA: Transforming growth factor-beta and extracellular matrix deposition in the kidney. *Contrib Nephrol* 107:140–145, 1994
38. Isaka Y, Fujiwara Y, Ueda N, Kaneda Y, Kamada T, Imai E: Glomerulosclerosis induced by in vivo transfection of transforming growth factor beta or platelet-derived growth factor gene into the rat kidney. *J Clin Invest* 92:2597–2601, 1993
39. Jones C, Buch S, Post M, McCulloch L, Liu E, Eddy A: Renal extracellular matrix accumulation in acute puromycin aminonucleoside nephrosis in rats. *Am J Pathol* 141:1381–1396, 1992
40. Eddy A: Expression of genes that promote renal interstitial fibrosis in rats with proteinuria. *Kidney Int* 49:49–54, 1996
41. Derynck R, Jarrettt J, Chen E, Eaton H, Bell J, Assoian R, Roberts A, Sporn M, Goeddel D: Human transforming growth factor-beta complementary DNA sequence and expression in normal and transformed cells. *Nature* 316:701–705, 1985
42. Wakefield L, Smith D, Flanders K, Sporn M: Latent transforming growth factor-beta from human platelets: A high molecular weight complex containing precursor sequences. *J Biol Chem* 263:7646–7654, 1988
43. Miyazono K, Hellman U, Wernstedt C, Heldin C: Latent high molecular weight complex of transforming growth factor-beta1: Purification from human platelets and structural characterization. *J Biol Chem* 263:6407–6415, 1988
44. Schultz-Cherry S, Lawler J, Murphy-Ullrich JE: The type 1 repeats of thrombospondin 1 activate latent transforming growth factor-beta. *J Biol Chem* 269:26783–26788, 1994
45. Schultz-Cherry S, Chen H, Mosher D, Misenheimer T, Krutzsch H, Roberts D, Murphy-Ullrich J: Regulation of transforming growth factor-beta activation by discrete sequences of thrombospondin 1. *J Biol Chem* 270:7304–7310, 1995-