The thrombospondin 1–TGF-β axis in fibrotic renal disease

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Introduction

Specific treatment of chronic progressive renal disease is very limited. During disease progression, extracellular matrix accumulation is the common hallmark of basically any renal process causing end-stage renal failure in man. Emerging evidence supports the concept of a major role for a thrombospondin 1 (TSP1)–TGF-β axis in scarring renal disease.

TGF-β is a potent cytokine affecting growth, differentiation and gene expression [1] that apparently needs to be tightly controlled. Lack of TGF-β as demonstrated in gene-deficient mice results in a severe generalized autoinflammatory response, developmental abnormalities, increased tumorigenesis, deficient wound healing, and early death. In contrast, an excess of active TGF-β causes enhanced tumour progression, as well as progressive fibrosis in multiple organ systems and suppression of the immune system. TGF-β is secreted by most cell types as a latent, inactive procycokine complex that needs to be activated extracellularly to bind to its receptors [2]. Within the latent procycokine complex, the mature active TGF-β protein is non-covalently bound to a dimer of its N-terminal pro-cycoketide, the so-called latency-associated protein (LAP), and variably to a latent TGF-β binding protein (LTBP).

In most in vitro systems or in normal tissues, very little TGF-β is present in its biologically active form. Since TGF-β protein and TGF-β receptors are widely expressed by numerous tissues and cells, the critical regulator of TGF-β-mediated action appears to be the activation process of the latent TGF-β molecule. This concept is supported by the observation 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 [3]. Studies demonstrated that denaturing agents such as pH changes, gamma irradiation, detergents or heat as well as reactive oxygen species, proteolysis by plasmin, calpain or cathepsin can activate TGF-β under in vitro conditions. Relatively little is known about TGF-β activation in inflammatory processes in vivo [1,2]. In an experimental pulmonary fibrosis model in mice, TGF-β activation was mediated via an integrin αvβ6-dependent mechanism [4] and other integrins may be involved as well [5,6].

TSP1 is a homeotrimeric multifunctional glycoprotein expressed by a variety of cell types including platelets, vascular smooth muscle cells and mesangial cells (MCs) and is frequently expressed at sites of inflammation and wound healing [7]. In vitro studies in various cell types have demonstrated multiple functions of TSP1 in modulating platelet aggregation, angiogenesis, cell proliferation, cell adhesion and migration, and TGF-β activity. As a typical matricellular protein, TSP1 is tightly regulated by cytokines such as PDGF, FGF-2 or TGF-β, and by various possible interactions with other cytokines, receptors and proteases. Its effect mainly depends on the local environment. These various biological activities of TSP1 relate to specific domains of the complex molecule including an N-terminal heparin binding region, a procollagen-like region with intermolecular disulphide bonds, three properdin-like type 1 repeats, three EGF-like type 2 repeats, a calcium-sensitive type 3 repeat, and a C-terminal globular domain. Multiple receptors such as heparin sulphate, calreticulin, LRP, CD36, αvβ3-integrin and CD47 as well as specific peptide sequences such the TGF-β-activating sequence RFK have been identified to be responsible for certain actions. Loss of TSP1 expression in TSP1 null mice causes a mild phenotype with predominant inflammatory lung disease [8]. The mild histological changes apparent in different organs of TSP1 null mice pups (mainly lung and pancreas) were very similar to those in TGF-β1 null mice pups [9] and could be reverted by treatment with a TSP1 corresponding peptide that activates TGF-β. Local overexpression of TSP1 in keratinocytes suppresses wound healing and granulation tissue formation in the skin of transgenic mice [10]. While the pronounced delay of wound closure in these TSP1 transgenic mice was accompanied by a
marked antiangiogenic effect and inhibition of fibroblast proliferation/migration, local TGF-β expression and activity was not examined.

**TGF-β in renal disease**

The role of TGF-β as a major profibrotic cytokine in various fibrotic diseases in multiple organ systems and in particular in experimental renal disease has been well established [11,12]. One of the best defined and examined experimental glomerulonephritis models in the rat, resembling typical features of human mesangial proliferative glomerulonephritis [13], is the anti-Thy1 model. In this model, a single injection of an anti-Thy1 antibody results in an acute, complement-dependent MC injury (days 0–2) with proteinuria, followed by an FGF-2- and PDGF-dependent MC proliferative response that is accompanied by a TGF-β-dependent overproduction of extracellular matrix proteins (days 3–10). TGF-β1 mRNA and protein are increased in the anti-Thy1 model [11]. Blocking of TGF-β1 action by injections with either a polyclonal anti-TGF-β1 antibody or with the proteoglycan decorin, a TGF-β1, -2 and -3 binding protein, as well as gene transfer techniques using antisense oligonucleotides against TGF-β1 or a decorin cDNA expression plasmid markedly reduce extracellular matrix accumulation [11,14,15]. 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 [16]. In addition, the association of TGF-β expression and renal disease progression as well as the profibrotic role of TGF-β has been documented in many different experimental renal disease models including diabetic nephropathy, the number one cause of kidney replacement therapy in the US. The group of Ziyadeh and Sharma [17,18] demonstrated short-term and long-term prevention of renal insufficiency and excess mesangial matrix expansion by treatment with an anti-TGF-β antibody in diabetic mice. Evidence for the central importance of TGF-β in mediating fibrosis in human kidney disease is well supported by the widespread correlation of TGF-β up-regulation with extracellular matrix excess in any type of human kidney disease [19].

**Thrombospondin 1 activates latent TGF-β**

Ten years ago, Murphy-Ullrich et al. [20] observed growth inhibitory effects of platelet TSP1 that were partially TGF-β dependent. Subsequently, it was demonstrated that TSP1 is complexed with TGF-β in platelet α-granules and that TSP1 stripped from TGF-β is able to convert the latent TGF-β procytokine to the biologically active form of TGF-β in cell culture.
and cell free systems [21]. For TGF-β activation, direct binding of TSP1 to the LAP and TGF-β protein is required forming an active ternary LAP–TSP1–TGF-β complex, while LTBP does not play a role. The failure of protease inhibitors to influence this process and the persistence of TGF-β activity within this LAP–TSP1–TGF-β complex suggests that binding of TSP1 to LAP–TGF-β mediates a conformational change within the LAP–TGF-β procytokine complex that abolishes a specific interaction required for latency. More detailed studies identified two important sites within the TSP1 molecule that are responsible for this complex interaction (Figure 1). One is the WxxW (WSHW, WSPW or WGPW) motif from the type I repeats of the TSP1 molecule that is used as the binding site to the active TGF-β domain [22]. Binding of this site seems to be essential to correctly orient the second TSP1 motif, the (K)RFK-sequence, allowing interaction with the correct complementary site on the latent TGF-β molecule, the N-terminal LSKL-sequence of the LAP [23]. This complex interaction leads to a conformational change probably within the LAP that allows the mature TGF-β protein to bind to its receptors still being incorporated in the ternary complex. Interestingly, TSP2 can bind to the LAP–TGF-β procytokine complex via its WxxW motif, but is unable to activate latent TGF-β, probably because of the lack of a (K)RFK sequence. Therefore, TSP2 can actually serve as a specific inhibitor of TSP1-mediated TGF-β activation (shown in a cell-free system), but whether this is actually relevant in in vivo situations has still to be determined. The LSKL sequence of the LAP is highly conserved in all five TGF-β isoforms and TSP1 also activates latent TGF-β/2, while other TGF-β isoforms still need to be tested. Nevertheless, some research groups reported failure of TSP1-mediated TGF-β activation in their systems [24,25]. The reason for these differences is unclear. They may relate to the local environment and additional factors being required for TSP1-mediated TGF-β activation under certain conditions. For example, the group of Khalil et al. described that activation of rat alveolar macrophage-derived latent TGF-β requires a complex interaction of plasmin with TSP1 and its receptor CD36 in vitro. This is likely to be important in vivo for bleomycin-induced pulmonary fibrosis, where a synthetic CD36 peptide abrogated TGF-β activation and tissue fibrosis [26,27].

**Thrombospondin 1 as a potential activator of latent TGF-β in renal cells in vitro**

The expression of TSP1 in cultured MC as an early response gene is induced by various cytokines such as PDGF, FGF-2 or TGF-β and by an increase in glucose concentration [28,29]. In parallel with TSP1, high glucose stimulated activation of latent TGF-β as well as matrix accumulation, as assessed by PAI-1, fibronectin or osteopontin [28,29]. In these studies, glucose-mediated TGF-β activation and matrix accumulation were inhibited using specific strategies to block TSP1–TGF-β interaction such as monoclonal anti-TSP1 antibodies, anti-TSP1 antisense oligonucleotides, or synthetic peptides (GGWSHW or LSKL). Furthermore, Wang et al. [30] demonstrated that glucose-mediated TSP1 regulation/TGF-β activation in cultured MC is mediated by an inhibition of the bioavailability of nitric oxide and subsequent nitric oxide-dependent signalling of the cGMP-dependent protein kinase. Glucose-mediated TSP1 expression was prevented by addition of either nitric oxide donors, a soluble guanulate cyclase inhibitor (ODQ), an inhibitor of cGMP-dependent protein kinase (Rp-8-pCPT-cGMP) or the nitric oxide synthase cofactor tetrahydrobiopterin.

**Thrombospondin 1 as a potential activator of latent TGF-β in renal disease in vivo**

While TSP1 may modulate its environment via TGF-β-dependent and TGF-β-independent mechanisms, there is increasing evidence in inflammatory renal disease for TSP1 being a major activator of TGF-β-promoted renal fibrosis (Figure 1). In human renal disease, TSP1 is predominantly expressed in glomerular and tubulointerstitial areas with most severe injury such as for instance in crescentic glomerulonephritis, but not in normal kidney or minimal change lesions [31]. In various experimental renal disease models resembling human diseases such as mesangial proliferative, membranous, minimal change or hypertensive nephropathy, TSP1 expression within the tubulointerstitium preceded and correlated with the degree of fibrosis [32]. In these renal disease models, TSP1 expression was spatially and temporally associated with increased TGF-β and could be used as an early predictor of the resulting degree of fibrosis. Since both the profibrotic function of TGF-β and glomerular de novo expression of TSP1 after injury is most convincingly shown in the anti-Thy1 model of mesangial proliferative glomerulonephritis [33], we first sought to inhibit TSP1-mediated activation of latent TGF-β in this well defined in vivo model system (C. Daniel, J. Wiede, H. C. Krutzsch et al., submitted for publication; C. Daniel, J. Wiede, J. Takabatake et al., submitted for publication). Similar to the approaches in cultured renal cells, de novo expression of TSP1 at sites of inflammation was either antagonized by transfer of phosphorothioate oligodeoxynucleotides (ODN) against TSP1 into renal glomeruli (C. Daniel, J. Wiede, J. Takabatake et al., submitted for publication) or by blocking the TSP1-mediated activation process of TGF-β using continuous intravenous infusion of specific peptides interfering with this interaction (C. Daniel, J. Wiede, H. C. Krutzsch et al., submitted for publication).

Successful inhibition of TSP1 expression in nephritic glomeruli after antisense ODN treatment, but not scrambled ODN therapy, was associated with a marked decrease in glomerular TGF-β activity as assessed by several methods (immunostaining for active TGF-β/TGF-β signalling molecules and bioassay for active
TGF-β). Antisense, but not scrambled, therapy against TSP1 almost completely abolished transcript expression of a typical TGF-β dependent gene such as ED-A-fibronectin in isolated glomeruli from nephritic rats and subsequently inhibited accumulation of several matrix proteins such as fibronectin and collagen. In addition, activation of MC to myofibroblasts resembling the profibrotic MC phenotype was also markedly inhibited by TSP1 antisense ODN treatment. Equivalent results regarding TGF-β1 levels. Reduced TGF-β1 have generally reduced serum and tissue TGF-β1 levels. Reduced TGF-β1 levels in these mice are associated with increased cell turnover and susceptibility to tumorigenesis in liver and lung [36], which has not been described for the TSP1 null mice. Therefore, therapeutic long-term strategies focusing on non-specific, systemic blockade of TGF-β1 ligand–receptor interactions may have problematic side effects due to the complex functions of TGF-β1 in vivo. In contrast, targeting TSP1-mediated activation of TGF-β1 as a therapeutic intervention for fibrotic kidney disease may have great promise because TGF-β1 is tightly regulated by cytokines in response to injury. The interaction of TSP1 with TGF-β1 is responsible for the major part of the glomerular matrix formation occurring in this model, but does not influence proliferation or macrophage accumulation. A therapeutic strategy inhibiting specifically only TSP1-mediated TGF-β1 activation in inflammatory disease as demonstrated may prove to be especially favourable given the known dual effects of TGF-β1 as a profibrotic as well as an anti-inflammatory cytokine. Further studies have to assess whether TSP1-mediated TGF-β1 activation is a general spread mechanism involved in various inflammatory renal diseases in man including diabetic and hypertensive nephropathy, or whether it is restricted to more limited situations.

In conclusion, the studies described above suggest TSP1 as a major activator of TGF-β1 in experimental glomerulonephritis (Figure 1). This activator of TGF-β1 is tightly regulated by cytokines in response to injury. The interaction of TSP1 with TGF-β1 is responsible for the major part of the glomerular matrix formation occurring in this model, but does not influence proliferation or macrophage accumulation. A therapeutic strategy inhibiting specifically only TSP1-mediated TGF-β1 activation in inflammatory disease as demonstrated may prove to be especially favourable given the known dual effects of TGF-β1 as a profibrotic as well as an anti-inflammatory cytokine. Further studies have to assess whether TSP1-mediated TGF-β1 activation is a general spread mechanism involved in various inflammatory renal diseases in man including diabetic and hypertensive nephropathy, or whether it is restricted to more limited situations.

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