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Thrombospondin-1 regulation of latent TGF-β activation: a therapeutic target for fibrotic disease

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Abstract

Transforming growth factor-β (TGF-β) is a central player in fibrotic disease. Clinical trials with global inhibitors of TGF-β have been disappointing, suggesting that a more targeted approach is warranted. Conversion of the latent precursor to the biologically active form of TGF-β represents a novel approach to selectively modulating TGF-β in disease, as mechanisms employed to activate latent TGF-β are typically cell, tissue, and/or disease specific. In this review, we will discuss the role of the matricellular protein, thrombospondin 1 (TSP-1), in regulation of latent TGF-β activation and the use an antagonist of TSP-1 mediated TGF-β activation in a number of diverse fibrotic diseases. In particular, we will discuss the TSP-1/TGF-β pathway in fibrotic complications of diabetes, liver fibrosis, and in multiple myeloma. We will also discuss emerging evidence for a role for TSP-1 in arterial remodeling, biomechanical modulation of TGF-β activity, and in immune dysfunction. As TSP-1 expression is upregulated by factors induced in fibrotic disease, targeting the TSP-1/TGF-β pathway potentially represents a more selective approach to controlling TGF-β activity in disease.

Keywords

thrombospondin-1; TGF-β; fibrosis; anti-fibrotic; latent TGF-β; diabetic nephropathy

Introduction

Transforming growth factor-β (TGF-β) is central to diseases involving elaboration of a fibrotic extracellular matrix (ECM), epithelial to mesenchymal transition and tumor metastasis, and immune dysregulation [1, 2]. However, TGF-β is essential for tissue

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Conflicts of interest:

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homeostasis and control of ligand expression, its multiple receptors, and complex downstream signaling mediators is critical for maintaining appropriate contextual action. Another regulatory checkpoint occurs through mechanisms that convert the latent precursor of TGF-β to its biologically active form, a process called activation [3, 4]. Non-covalent binding of the N-terminal latency associated peptide (LAP) to the C-terminal mature domain prevents TGF-β receptor binding and this interaction must be disrupted for TGF-β to be active. Only a small fraction of total TGF-β is biologically active. Latent TGF-β can be activated through proteolysis (including plasmin and matrix metalloproteinases); binding to integrins $\beta_v\beta_1$, $\beta_v\beta_3$, $\beta_v\beta_5$, $\beta_v\beta_8$, or $\beta_v\beta_6$ in concert with mechanical forces due to ECM stiffness and/or cytoskeletal forces, or in the case of $\beta_v\beta_3$, and $\beta_v\beta_8$, through localizing matrix metalloproteinases to the latent complex; through viral or oxidative modifications of the latent complex; or by binding to the secreted and extracellular matrix (ECM) protein thrombospondin 1 (TSP-1) [4–13]. The primary mechanism of latent TGF-β activation varies with cell type, tissue, and disease milieu. Blockade of a major disease-related activation mechanism typically attenuates the effects of disease-induced TGF-β activity and thus represents a novel therapeutic strategy to attack disease-induced activity and spare homeostatic TGF-β.

Integrin and TSP-1 dependent activation have been the most widely studied mechanisms of latent TGF-β activation. This review will focus on TSP-1 control of latent activation as a regulatory mechanism and discuss relevant diseases in which this mechanism is operative, with an emphasis on TGF-β in fibrotic complications of diabetes in the kidney and in the heart. We will also discuss the involvement of TSP-1 in controlling TGF-β activity in various models of liver fibrosis and in the hematologic cancer, multiple myeloma. Finally, we will briefly address emerging evidence for the role of the TSP-1/TGF-β axis in other disease indications and approaches to therapeutic targeting of the TSP-1/TGF-β pathway.

Thrombospondin 1 (TSP-1) activates latent TGF-β

TSP-1 is a multi-functional matricellular ECM and secreted protein, abundant in platelet β-granules, with widely upregulated expression in tissue injury and repair [14, 15]. TSP-1 has structural domains with multiple specific receptors/binding partners and cellular functions [16]. TSP-1 is a major regulator of TGF-β activation, but also has TGF-β-independent functions in hemostasis, cell adhesion, migration, growth factor (EGF, VEGF, FGF) regulation, and inhibition of angiogenesis and nitric oxide signaling [14].

A number of years ago we observed that preparations of human TSP-1 purified from pools of thrombin-stimulated platelets contained biologically active TGF-β1 that co-migrated with TSP-1 in gel permeation columns, suggesting direct binding between the two proteins [17]. In more interesting further studies, we showed that purified TSP-1, as well as certain proteolytic or recombinant fragments of the protein can bind to either the small or large form of the purified latent TGF-β complex and to latent TGF-β secreted by cultured cells. TSP-1 binding to the latent complex converts latent TGF-β to its biologically active form through a non-proteolytic mechanism [18, 19]. Subsequent studies identified the KRFK sequence in the TSP-1 type 1 repeats as critical for latent TGF-β activation [20, 21]. The KRFK sequence in the 2nd TSP-1 type 1 repeat binds to a conserved sequence, LSKL, in the LAP,

which disrupts LAP-mature domain interactions to expose the receptor binding sequences, rendering TGF-β capable of signaling (Figure 1) [22, 23]. TSP-2 lacks the KRFK sequence and does not activate TGF-β and in fact, can act as a competitive antagonist of TSP-1 mediated TGF-β activation [21, 24]. We identified the LSKL sequence in the LAP as a critical determinant of latency, a result that has been confirmed biochemically and by the crystal structure of the latent complex [22, 25, 26]. In addition, we identified sites of interaction between the tryptophan-rich motifs in the type 1 repeats of TSPs 1 and 2 and the latent complex, which are important for binding but not sufficient for activation [27]. TSP-1 binds to latent TGF-β to activate TGF-β in solution, at the cell surface, or in the extracellular milieu in a manner independent of proteases [7]. The molecular nuances of TSP-1-latent TGF-β interactions and the activation process were discussed extensively in a previous review and the reader is encouraged to consult this article for additional details [7].

The LSKL peptide is a competitive antagonist that inhibits TSP-1-TGF-β activation by preventing the interaction of TSP-1's KRFK sequence with the LAP of latent TGF-β (Figure 1) [23]. This antagonist tetrapeptide has been widely used by our lab and others, along with *thbs1* (TSP-1) null animals, to establish TSP-1 as a primary regulator of TGF-β bioactivity in numerous fibrotic diseases, including diabetic nephropathy and cardiomyopathy [7, 28–31]. A summary of animal studies from numerous labs in which LSKL has been used to antagonize TSP-1-dependent TGF-β activation is found in TABLE 1. In addition, there are diverse indications for TSP-1 involvement in latent TGF-β activation based on either in vitro studies, models using *thbs1* deficient animals, or blocking antibody or other antagonist peptide studies as presented in [7]. Some more recent indications for involvement of the TSP-1/TGF-β axis in disease will be discussed.

TSP-1 activation of TGF-β isoforms

The LSKL sequence is conserved in the LAP regions of all three mammalian isoforms of latent TGF-β, suggesting that TSP-1 can activate all three isoforms [23]. TGF-β1 is considered the primary isoform driving fibrosis and much of the focus on TSP-1 control of TGF-β1 activation has been centered on investigation of its role in various fibrotic diseases. Indeed, there is ample evidence for TSP-1 activation of latent TGF-β1 as discussed here and elsewhere [7]. Initial evidence for a physiologic role for TSP-1 in regulating activation of latent TGF-β1 resulted from observations that *thbs1* null mice partially phenocopies the TGF-β1 null mice, albeit with some distinctions and a less severe phenotype than the TGF-β1 null mice [30]. These studies demonstrated that treatment of neonatal mice with a TSP-1 peptide that activates latent TGF-β (KRFK) could partially rescue the TGF-β1 null phenotype and the LSKL blocking peptide could partially replicate this phenotype in wildtype neonatal mice [30]. With respect to the other mammalian isoforms, little is known about mechanisms involved in activating latent TGF-β3 in vivo, although in vitro studies indicate that MMPs and integrin $\beta_v\beta_6$ can activate TGF-β3: furthermore, TGF-β3 and βvβ6 and βvβ8 knockout mice all have cleft palate, suggesting a role for integrin-dependent activation [13, 32–34]. The ability of TSP-1 to activate latent TGF-β3 has not been directly tested and nor has its co-expression in various tissues been examined. In contrast, there is evidence that TSP-1 can activate latent TGF-β2 and that activation of latent TGF-β2 can be blocked by LSKL both using recombinant latent TGF-β2 and in hypoxia-stimulated

endothelial cells [23, 35]. Furthermore, murine antigen presenting cells use TSP-1 to activate latent TGF-β2, a process that is important for induction of Foxp3+ T regs [36]. In this system, TSP-1 binding to CD36 was required for TGF-β activation and the authors suggest that TSP-1 represents a mechanism for controlling TGF-beta activation at mucosal surfaces [36]. Interestingly, unlike in the antigen presenting cells, TSP-1 regulates expression of latent TGF-β2 at the protein level in lactate-treated glioma cells with TSP-1 knockdown reducing both TGF-β2 protein and Smad phosphorylation [37]. This is similar to the observation that LSKL reduces TGF-β2 mRNA in hypoxic endothelial cells and likely reflects auto-stimulatory effects of active TGF-β2 on its own expression [35]. Similarly, TSP-1 is known to induce TGF-β2 expression in vascular smooth muscle cells [38]. As the LAP of TGF-β2 lacks the integrin binding RGD sequence and this isoform is not activated by integrins [33], together these data suggest that TSP-1 is a major regulator of activation of the latent TGF-β2 isoform. The fact that there is little overlap in phenotypes between *thbs1* null and TGF-β2 null mice suggests that TSP-1 control of latent TGF-β2 activation would occur primarily under post-natal conditions, rather than during development [30, 39]. For clarity, “TGF-β” will be used to designate either TGF-β1 in disease models in which this isoform predominates or which have not rigorously addressed isoform specificity. If the TGF-β isoform is known, it will be specifically designated.

TGF-β is a therapeutic target in Diabetic Nephropathy

TGF-β is central to development of diabetic nephropathy. TGF-β1 is a key mediator of progressive diabetic nephropathy by inducing glomerular hypertrophy with mesangial matrix expansion, renal tubular injury and interstitial fibrosis, and alterations in podocyte slit barrier function, leading to proteinuria [40–42] [43]. TGF-β inhibits epithelial repair by blocking growth, promoting apoptosis, and by stimulating epithelial plasticity [44]. The diabetic milieu (hyperglycemia, the renin-angiotensin system, and increased reactive oxygen species) stimulates increased TGF-β activity. In addition, increased expression of TGF-β in glomeruli and tubules is documented in both diabetic patients and animal models [45]. Thus, it is well-established that TGF-β is a therapeutic target for diabetic nephropathy [40, 46–51].

TSP-1 regulates latent TGF-β activation in diabetic complications

There are multiple lines of evidence from clinical specimens, cell culture, and animal models supporting the premise that TSP-1-mediated latent TGF-β activation is a critical factor in the development of diabetic nephropathy. Factors associated with increased TGF-β activity in diabetes stimulate TSP-1 expression. Oxidative stress under high glucose conditions increases TSP-1 expression by mesangial cells due to a decrease of nitric oxide-protein kinase G-mediated transcriptional repression and increased expression of the transcription factor USF2 [52, 53]. Glucose also increases podocyte TSP-1 expression [54]. Angiotensin II increases TSP-1 expression by mesangial cells and cardiac fibroblasts *in vitro*, which results in increased TGF-β activity [53, 55, 56]. TSP-1 protein is increased in the glomeruli of patients with both types 1 and 2 diabetic nephropathy and its expression correlates with increased TGF-β activity [57, 58] and with proteinuria in patients with type 2 diabetes [57]. *Thbs1* gene expression is increased in mesangial cells isolated from *db/db* mice with type 2 diabetic nephropathy [59]. TSP-1 was identified as a biological marker of

obesity and metabolic syndrome in human adipose tissues and serum [60]. Glucose-induced TGF-β activity in rat mesangial cells is blocked by LSKL and WSHW peptide antagonists and by an anti-TSP1 antibody [55, 61]. Glycated albumin stimulates increased TSP-1 expression and tubular hypertrophy: antibodies to TSP-1 block the increase in TGF-β activity and tubular hypertrophy [62]. The increased TGF-β activity in angiotensin II stimulated mesangial cells is blocked by TSP-1-TGF-β antagonist peptides [56]. Moreover, streptozotocin-treated *thbs1* null mice do not develop glomerulosclerosis or podocyte loss [31] and conversely, mice with induced expression of the transcription factor USF2 have increased TSP-1 expression, TGF-β activity, and renal fibrosis [63].

TSP1 and obesity-related diabetic complications

Type 2 diabetes and hypertension are the two leading causes of end stage renal disease (reviewed in [64]). Obesity is a major risk factor for type 2. There are overlapping mechanisms underlying kidney disease in diabetes and obesity, which include hyperfiltration, the influence of inflammatory cytokines released from adipose tissue, and activation of the renin-angiotensin system [64–66]. TGF-β is elevated in patients with type 2 diabetes and in at least two mouse models of diabetic nephropathy (*db/db*, KK-Ay) [67–69]. Anti-TGF-β antibody reduces glomerulosclerosis and reverses glomerular basement membrane thickening in the *db/db* model of type 2 diabetic nephropathy [67, 70]. TSP-1 protein is increased in type 2 diabetic nephropathy and its expression correlates with increased TGF-β activity and with proteinuria in patients with type 2 diabetes [57, 58]. TSP-1 was the most highly upregulated protein in mesangial cells from *db/db* mice [59]. TSP-1 is also implicated in renal dysfunction and fibrosis in a mouse model of high fat diet induced obesity downstream of leptin signaling [71].

Interestingly, leptin stimulates TSP-1 expression, and TSP-1 deficient mice on a high fat diet are protected from renal fibrosis and albuminuria and have reduced TGF-β signaling [71]. *In vitro* leptin treatment of TSP-1-deficient mesangial cells failed to stimulate increased TGF-β activity or fibronectin and type IV collagen expression [71]. This is paradoxical to models of type 2 diabetic nephropathy in which either leptin or its receptors are genetically deleted (*ob/ob, db/db*, respectively).

Both TGF-β and TSP-1 have been shown to play causal roles in insulin resistance and obesity-related renal fibrosis, although there is evidence for both TGF-β-dependent and independent roles of TSP-1 [72–75]. It remains to be determined whether blocking TSP-1-TGF-β activation would lower insulin resistance.

TSP-1-TGF-β antagonists in diabetic complications

In Akita C57BL/6J-*Ins2*^{Akita} mice, we showed that antagonism of TSP-1-dependent TGF-β activation by intraperitoneal injection of LSKL peptide (30 mg/kg, 3x/week for 15 weeks) decreased proteinuria and tubulointerstitial fibronectin expression, increased nephrin expression, and reduced TGF-β activity in the kidney and urine [28]. It is notable that LSKL reduced both fibrosis and proteinuria, since studies with a monoclonal antibody to TGF-β or knockout of Smad 3 reduced fibrosis without attenuating proteinuria [67, 76]. Data suggest

TGF-β signaling through the ALK1 receptor, Smad 1/5 pathway might be critical for TGF-β-induced proteinuria [77]. It is possible that the TSP-1 antagonist by blocking latent TGF-β activation could impact both the classical and alternate Smad pathways. In addition to blocking the development of fibrosis and improving renal function in the diabetic kidney, LSKL peptide reversed myocardial fibrosis and improved left ventricular function through reducing TGF-β activity in hypertensive diabetic rats when treatment was initiated 6 weeks after disease induction [29]. This is an important finding since antagonist peptide treatment began at a time when fibrotic changes were already detectable in the left ventricle, suggesting that blocking the TSP-1-TGF-β pathway might be an effective anti-fibrotic strategy in patients with early diabetic cardiomyopathy.

TSP-1 antagonists and the renin-angiotensin system

Antagonists of the renin-angiotensin system are the standard of care for diabetic nephropathy. However, 20% of patients progress to end stage renal disease despite treatment (reviewed in [78]). Although the renin-angiotensin system drives diabetic nephropathy through multiple mechanisms, it directly impacts both TSP-1 expression and TGF-β expression and activity. We showed that angiotensin II stimulates TSP-1 expression by rat mesangial cells and that losartan blocks angiotensin II stimulation of TSP-1 [56]. The TSP-1 antagonist LSKL reduces TGF-β activity, but not TGF-β protein expression, in rat mesangial cells stimulated by angiotensin II [56]. Valsartan attenuates TSP-1 and TGF-β expression and TGF-β protein and Smad signaling in aortas of diabetic rats [79]. Despite some overlap in their targets, synergy between TGF-β antagonists and renin-angiotensin inhibitors has been observed in animal models of diabetic nephropathy [80, 81] [82]. It remains to be determined experimentally as to whether combinations of TSP-1-TGF-β antagonists with renin-angiotensin antagonists would provide additional benefit.

Other TGF-β activation mechanisms in diabetic complications

Integrin-dependent TGF-β activation also represents a potential target for diabetic nephropathy [83–85]. There is a recently completed phase 2 trial (NCT02251067) for diabetic nephropathy using an antibody (VPI-2690B) that blocks ligand binding to the $\beta_v\beta_3$ integrin and integrin activation: this antibody also reduces TSP-1 and TGF-β expression [86, 87], although it is not known whether this antibody directly blocks TGF-β activation. Another integrin antagonist, the cyclic peptide that targets $\beta_v\beta_3$ and $\beta_v\beta_5$, Cilengitide, recently failed in trials of aggressive glioblastoma and importantly, there was no correlation of p-Smad 2 levels with Cilengitide treatment in this disease for reasons that are not clear [88]. There is a small molecule inhibitor of $\beta_v\beta_1$ (C8) which blocks fibrosis in several organs including the renal unilateral ureteral obstruction (UUO) model, although the relevance of this integrin to diabetic nephropathy has not yet been established [89, 90].

The role of TGF-β in liver fibrosis

TGF-β is a central regulator of chronic liver disease through induction of fibrogenic responses [91–95]. Although infiltrating macrophages are a source of TGF-β, hepatic stellate cells are a significant source of TGF-β in liver fibrosis. TGF-β stimulates induction

of myofibroblast-like properties of hepatic stellate cells to produce extracellular matrix, leading to fibrosis. Although TGF-β inhibits hepatocyte proliferation under basal conditions, it has pro-oncogenic properties during malignant progression through stimulating epithelial to mesenchymal transition, cell survival and migration, and reduced immune surveillance.

TSP-1 control of latent TGF-β activation in liver fibrosis

TSP-1 expression is increased in human liver disease with the *THBS1* gene identified as part of a characteristic gene signature of chronic liver disease, including alcoholic and NASH cirrhosis, in humans and in mouse models of liver fibrosis induced by carbon tetrachloride or DDC [96]. In vitro studies show that bile acids increase expression of TSP-1 by hepatocytes, resulting in increased TGF-β signaling in co-cultured hepatic stellate cells [97]. Both TSP-1 and TGF-β are increased in congenital hepatic fibrosis [98]. TSP-1 regulated TGF-β activation prevented hepatocyte proliferation and liver regeneration after partial hepatectomy in *thbs1* deficient mice [99] and TSP-1 induction by obstructed portal flow in mice is thought to lead to TGF-β-dependent liver atrophy [100]. TSP-1 has been shown to regulate latent TGF-β activation in animal models of liver fibrosis and in cell culture models (reviewed in [101]). Treatment of rats with the TSP-1 antagonist peptide LSKL prevented TGF-β activation and reduced liver fibrosis in the dimethylnitrosamine model [102]. TSP-1 is required for TGF-β signaling in both cultured hepatocytes and hepatic stellate cells, which is blocked by LSKL peptide [103, 104]. Interestingly, TSP-1-dependent latent TGF-β activation might play a role in hepatitis C induced fibrosis and carcinogenesis as the hepatitis C virus core protein induces TSP-1 expression by hepatocytes to increase active TGF-β and LSKL peptide blocks hepatitis C core protein activation of TGF-β [105]. Administration of LSKL peptide immediately after injury also accelerated liver regeneration in mice following partial hepatectomy through blocking TGF-β activation and signaling [106]. Both the TGF-β1 and the TGF-β2 isoforms are upregulated in mouse models and in human tissues with liver fibrosis and also hepatocellular carcinoma [107]. This is interesting since TSP-1 can activate both the TGF-β1 and β2 isoforms of latent TGF-β, whereas TGF-β2 is not activated by integrin-dependent mechanisms.

Other diseases with a role for TSP-1-dependent latent TGF-β activation

Since our discovery of the role of TSP-1 in mediating latent TGF-β activation, identification of the LAP sequence LSKL as an antagonist of binding and activation and demonstration of its activity in vivo, studies from multiple labs using LSKL in a diverse array of diseases have established the involvement of TSP-1 in a particular disease process [18, 23, 30]. Many of these in vitro and in vivo studies were summarized in a previous review [7]. Here we will discuss some more recent findings regarding disease models in which TSP-1 appears to be important for controlling latent TGF-β activation.

Multiple Myeloma

Multiple myeloma (MM) is a systemic cancer of malignant plasma cells. TGF-β has important roles in mediating myeloma cell-bone marrow stromal cell adhesion, activation of osteolytic pathways, and immune dysfunction [108–111]. TSP-1 expression is increased in the bone marrow plasma of MM patients [112, 113]. In a mouse xenograft model using

heparanase expressing human MM cells, treatment with either LSKL or a novel compound based on LSKL, SRI31277, reduced tumor burden, TGF-β signaling in the bone marrow, host IL-6 production, and osteolytic bone disease [114]. Interestingly, SRI31277 both reduced osteoclasts and increased osteoblasts on marrow bone surfaces, consistent with previous in vitro observations that TSP1 blocks osteogenic differentiation of mesenchymal stem cells and that LSKL can reverse this blockade to promote osteogenic differentiation [115]. Cocktails of various drugs are the standard of care in myeloma. Our studies showed that SRI31227 has added benefit when combined with the proteasome inhibitor, bortezomib, but there was no additional benefit when SRI31277 was combined with dexamethasone [114].

Pulmonary arterial hypertension, flow-mediated arterial stiffening, cardiomyopathy

Both TSP-1 and TGF-β are upregulated in pulmonary arterial hypertension due to chronic hypoxia, Schistosomiasis, and in scleroderma: recent studies show that *thbs1* knockout or treatment with the peptide antagonist LSKL protected against development of pulmonary hypertension due to hypoxia or Schistosome infection and also reduced active TGF-β [116]. In models of disturbed arterial flow leading to arterial stiffness in aged mice, TSP-1 and markers of pro-fibrotic TGF-β pathways were upregulated: in *thbs1* knockout mice or mice treated with LSKL via intraperitoneal administration and subjected to disturbed flow, there was reduced arterial collagen, connective tissue growth factor, and stiffening [117]. Other studies show that LSKL blocks the development of endomyocardial fibrosis in mice with genetic deletion of inhibitor of DNA binding 1 and 3 (Id1 and Id3), a suppressor of TSP-1 expression [118].

Fibrotic diseases with a biomechanical component

Although mechanotransduction-dependent activation of latent TGF-β is considered to occur through integrin and cytoskeletal-dependent mechanisms that induce sufficient force to unfold the straightjacket domain of LAP [9–11, 119], TSP-1 expression is increased by mechanical forces in a number of tissues as described here, raising the possibility that that mechanical forces also play a role in stimulating TSP-1-mediated latent TGF-β activation. An early observation showed that mechanical stretch of neonatal sheep lungs increased TSP-1 expression and TGF-β activity [120]. TSP-1 was also shown to be important for shear-stress activation of TGF-β induced by stirring of platelets [121]. In scleroderma, TSP-1 is increased in the plasma and dermis of patients [122, 123] and both hypoxia and TGF-β induce TSP-1 expression [124]. Furthermore, TSP-1 mRNA levels are one of a 4-gene signature biomarker that is predictive for disease activity in patients with diffuse cutaneous systemic sclerosis [125]. Stiffening of the skin is a characteristic of scleroderma and in vitro studies showed that force-loaded skin fibroblasts increase TSP-1 expression [126]. In addition, pre-treatment of fibroblasts with LSKL and then with active TGF-β reduced TGF-β-dependent fibroblast contractility, presumably by preventing autocrine TGF-β activation by TSP-1, with the effects of LSKL more pronounced in skin fibroblasts derived from scleroderma patients than from normal controls [126]. Although there is no direct evidence for TSP-1 control of TGF-β activation in scleroderma obtained through the use of specific TSP-1 antagonists in animal models, recent evidence obtained in the bleomycin

model of systemic sclerosis using the anti-fibrotic glycyrrhizin shows that TSP-1 levels, TGF-β signaling, and fibrosis were reduced [127].

Glaucoma is another disease in which biomechanical forces due to increased intraocular pressure and possibly intrinsic tissue responses are thought to contribute to disease progression [128, 129]. Increased intraocular pressure in glaucoma affects ECM remodeling in the trabecular meshwork with increased resistance to outflow and also remodeling of the lamina cribrosa, the collagenous tissue at the optic nerve head, which leads to optic neuropathy [130]. TSP-1 is increased by mechanical strain of cells isolated from the lamina cribrosa, and TSP-1 is also increased in the trabecular meshwork of eyes from patients with glaucoma [131–135]. Both TGF-βs 1 and 2 are increased in glaucoma and are important for the pathologic remodeling of these ocular tissues under elevated intraocular pressure [136, 137]. Interestingly, the TGF-β2 isoform, which is not activated by integrins, is widely expressed in glaucoma, suggesting either TSP-1 or other non-integrin dependent activation mechanisms could be important in glaucoma.

For both scleroderma and glaucoma—fibrotic diseases in which biomechanical alterations are a key characteristic, additional studies are needed to determine whether increased TSP-1 expression in response to mechanical strain contributes to latent TGF-β activation directly. It also remains unknown how mechanical forces might affect TSP-1 folding, presentation at the cell surface, or release from the ECM to favor binding to and activation of the latent TGF-β complex.

Immunotherapy modulators

TGF-β is immunosuppressive through induction of T regs and M2 macrophages and impairment of dendritic cell maturation and NK cell activity [138–140]. Based on these data, there is interest in using TGF-β antagonists to enhance anti-tumor immunotherapies and to augment bone marrow reconstitution following stem cell transplant [141–143]. Integrin $\alpha_v\beta_8$ expressed on dendritic cells is known to activate latent TGF-β and stimulate Th17 and T reg development, thereby influencing autoimmune disease pathogenesis [144–147]. Expression of $\alpha_v\beta_8$ on T regs also regulates TGF-β activity [148]. Despite the importance of integrins, there are also multiple lines of evidence showing that TSP-1 plays a role in TGF-β activation in the context of immune cells. TSP-1 null mice have reduced Th 17 cells and IL-17 levels due to decreased TGF-β activity [8, 149]. TSP-1 is expressed by T cells and accumulates on the T cell surface with latent TGF-β. In addition, TSP-1 activates latent TGF-β2 expressed by antigen-presenting cells to control T reg development in ocular tissues [36]. There is also evidence that B cell-derived TSP-1 regulates latent TGF-β activation on dendritic cells [150]. TSP-1 can also inhibit early NK proliferation in a manner blocked by anti-TGF-β neutralizing antibodies, suggesting a role for TSP-1 in latent TGF-β activation by human NK cells [151]. Moreover, adipose-derived stem cells reduced autoimmune colitis by inducing T regs through TSP-1 dependent TGF-β activation, but independent of integrin α_v [152]. Studies also suggest that TSP-1 regulated latent TGF-β activation, which can be blocked by LSKL, plays a role in T cell activation following renal transplant [153]; in immune tolerance in glioma [154]; in pigmented epithelial cell generation of T regs in ocular tissues [155]; and in cell senescence [156]. Clearly, additional studies are needed to

better understand the roles of integrin and TSP-1-dependent TGF-β activation mechanisms in different immune contexts. Nonetheless, these data suggest that TSP-1 antagonists might have potential therapeutic uses to optimize immunotherapy treatments through controlling TGF-β action.

Possible roles of TSP-1 receptors

It is possible that in some diseases, multiple activation mechanisms can control latent TGF-β activation by differing cell types and at different stages of disease progression. Alternately, there can be co-operative mechanisms that employ aspects of multiple activation mechanisms. For example, some integrins and MMPs can cooperate to regulate latent TGF-β activation [169]. There is also evidence that CD36-bound TSP-1 on bleomycin activated alveolar macrophages or on antigen presenting cells can activate latent TGF-β, but plasmin activity is required, at least in the former case [36, 170]. TSP-1 also binds to a number of different integrins and we observed that an antibody to the $\beta_v\beta_3$ integrin blocks TSP-1 activation of latent TGF-β in breast cancer cells and others showed that VP1-2690B antibody to integrin subunit β_3 decreases TSP-1 expression [86, 171]. TSP-1 binding to integrins or other TSP-1 receptors, as in the case of macrophage or antigen presenting cell CD36, might serve to localize TSP-1 and the latent complex at the cell membrane to enhance activation in the vicinity of TGF-β receptors. Although purified TSP-1 can activate recombinant latent TGF-β in a test tube in the absence of cells [114], in the cellular context, other stabilizing or localizing factors such as TSP-1 binding receptors or other cell surface or soluble molecules might be involved that could either favor or hinder TSP-1 availability for binding to the latent complex. Interestingly, in the absence of the TSP-1 receptor, CD47, there are elevated levels of both TSP-1 and active TGF-β following thermal injury, raising the possibility that signaling downstream of CD47 antagonizes the TSP-1/TGF-β pathway or possibly TGF-β signaling itself [172]. However, antibodies to integrin-associated protein (CD47) blocked activation of TGF-β in estrogen-depleted breast cancer cells in a system in which TSP-1 associated with the cell surface is increased and in which TGF-β activity is reduced by a blocking antibody (Mab133) and a blocking peptide [171]. Clearly, additional studies are needed to elucidate the role of TSP-1's complex interactions with cells on its ability to control latent TGF-β activation.

Relationship to integrin dependent activation

It is unknown whether integrin antagonists with anti-fibrotic activity also affect TSP-1 dependent TGF-β activation or whether combining TSP-1 and integrin antagonists would have additional therapeutic benefit. Double knockout mice that lack both *thbs1* and the integrin β6 subunit have a more severe inflammatory phenotype than either single knockout mouse. Also, the β6 single knockout mice have increased inflammation and tumorigenesis as compared to the *thbs1* knockout mice [173]. Curiously, the double knockout mice had increased staining of phosphorylated Smad2/3 in hyperplastic lung epithelium as compared to wild type mice for reasons that are not clear. Single knockout mice were not examined in these studies, although single knockout mice would be predicted to have reduced TGF-β signaling based on previous studies [6, 30, 173]. It is not definitively known if both TSP-1 and integrin-dependent activation can be operative in the same tissue and disease milieu.

Interestingly, there are several disease indications in which both integrin antagonists and TSP-1/TGF-β antagonists have been shown to reduce TGF-β activity and fibrotic disease: examples include the unilateral ureteral obstruction model of renal fibrosis, liver fibrosis, pulmonary fibrosis, and T cell regulation [6, 89, 102, 144, 146, 148, 149, 151, 152, 170, 174–177]. It has largely been assumed that blockade of a particular pathway with resultant attenuation of disease indicates that only that one process is critical for disease pathogenesis. However, it is possible that two independent pathways could control latent TGF-β activation in a particular organ or disease. For example, each pathway might differentially regulate activation by distinct cell populations or at different stages of disease progression. Both or multiple pathways could be required for full disease progression, given the cross-talk between cell populations in fibrosis, so that attenuation of one pathway is sufficient to attenuate disease. However, both integrin and TSP-1/TGF-β antagonists can affect similar cell populations such as myofibroblasts to reduce TGF-β signaling, so other explanations are likely and this remains an important question for the fibrosis field. In contrast to diseases in which the TGF-β1 isoform predominates, it is likely that TSP-1 is a major factor in TGF-β2-mediated diseases as integrins do not activate this isoform.

The lack of a complete phenocopy of *thbs1* null mice with TGF-β1–3 null mice has been used as an argument to suggest that TSP-1 is not a primary regulator of latent TGF-β activation. It should be noted that these phenotypes reflect developmental alterations and cannot predict the roles of these activation mechanisms in diseases processes that occur post-natally or in the adult. TSP-1 is a matricellular extracellular matrix protein and one of the defining characteristics of matricellular proteins is that knockout phenotypes at birth are mild with more robust phenotypes appearing as the animal ages or is exposed to stresses or in disease [15, 178]. This concept is consistent with multiple lines of evidence showing a role for TSP-1 in controlling latent TGF-β in various diseases in which TSP-1 expression is upregulated, but with only mild developmental phenotypes. Furthermore, TSP-1 binds multiple integrins and other receptors to regulate diverse biologic function such as cell adhesion, migration, apoptosis, VEGFR and nitric oxide signaling, angiogenesis, vascular barrier function, and inflammation in addition to regulation of TGF-β activation [179]. Thus, any *thbs1* knockout phenotype likely reflects a complex interplay of pathways altered by the loss of TSP-1 function. Nonetheless, more direct assessment of possible co-activity between integrin and TSP-1 mediated control of latent TGF-β activation through use of specific antagonists rather than via genetic approaches is needed.

Approaches to therapeutic control of TGF-β in disease and potential role for TSP-1/TGF-β antagonists

Although TGF-β is a significant player in fibrotic diseases, metastatic progression, and immune dysfunction, it is nonetheless critical for homeostasis. Genetic ablation of TGF-β, its receptors, or its signaling mediators can result in developmental defects, unremitting inflammation, and an increased incidence of carcinomas [157, 158]. Current anti-TGF-β antagonists target the molecule, receptors, or signaling mediators and provide no mechanism for distinguishing homeostatic from disease-induced TGF-β activity. This raises the potential for adverse effects with the use of global inhibitors such as monoclonal antibodies

to ligand or receptor kinase inhibitors as seen in long-term animal studies and more critically, in clinical trials [159–161]. In diseases such as diabetes, chronic treatment will be required and thus a targeted approach to reducing disease-specific TGF-β activity would potentially increase therapeutic benefit [1, 2, 138, 162]. To date, the use of the TSP1-TGF-β antagonists in animal models has not induced adverse events such as inflammation or tumorigenesis. Mice treated intermittently with LSKL (15 weeks, 3x/week i.p.) or continuously over 4 weeks with SRI31277 did not show inflammation or unrelated tumors [29, 114]. These findings are consistent with studies showing increased GI tumors and inflammation in mice with dual mutations that reduce basal TGF-β activity and prevent activation by integrin β_8, but no increase in tumors was observed in dual mutant mice with *thbs1* knockout [163]. Although TSP-1 deficient mice have modest inflammation, this could be due to TGF-β independent functions of TSP-1and it is not replicated by LSKL or SRI31277 [30, 164]. Importantly, systemic administration of LSKL peptide did not impair wound healing in diabetic *db/db* mice [28]. In addition, SRI31277 displayed no off-target activity in CEREP receptor binding assays (Suto M.J., Murphy-Ullrich, unpublished data). Although these multiple animal studies suggest that chronic use of TSP-1-TGF-β antagonists would be safe, there might be situations in which TSP-1-TGF-β activation is beneficial and in which blockade could lead to adverse events, as in a mouse model of pre-formed aneurysms [165].

In addition to the risks of globally attenuating TGF-β activity, results from clinical trials with global inhibitors of TGF-β have failed to show efficacy. Despite abundant evidence for the importance of TGF-β in diabetic nephropathy, clinical trials with monoclonal antibodies to TGF-β1 have not demonstrated therapeutic benefit. TGF-β antagonists (monoclonal antibodies GC-1008 and LY2382770) are no longer in clinical trials for diabetic nephropathy due to corporate decisions or lack of phase 2 efficacy, respectively [51]. A recent phase 2 study using a humanized anti-TGF-β1 antibody in combination with renin-angiotensin inhibitors was stopped early for lack of efficacy [161]. The anti-TGF-β antibody, 1D11, when tested in a mouse model of pulmonary fibrosis, o failed to reduce pulmonary fibrosis, but exacerbated inflammation [166]. There is an emerging consensus that global inhibition of TGF-β, particularly through use of ligand-targeted monoclonal antibodies, is not effective. The reasons are not clear, although observations of reduced efficacy with higher doses of antibody, coupled with adverse effects in monkeys, limit dosing intensity in chronic diseases [161, 167, 168]. It is possible that molecules as large as antibodies do not have sufficient tissue penetrance and access to the ligand, especially as latent TGF-β can be sequestered in the ECM or at the cell surface.

Conclusion

In light of clinical failures with global TGF-β antagonists, newer, more targeted approaches to controlling disease-related TGF-β activity are needed. Given the relative disease-restricted utilization of TGF-β activation mechanisms, antagonists of disease-specific TGF-β activation pathways potentially represent a more therapeutically effective means of targeting TGF-β in fibrotic diseases. Ideally, the antagonist should target only the TGF-β activating function of the inducing molecule. As we have described, antagonists of the TSP-1/TGF-β

pathway could be suitable drug candidates for fibrotic and other diseases involving pathologic TGF-β activation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ECM	extracellular matrix
LAP	latency associated peptide
MM	Multiple Myeloma
NK	Natural Killer
TSP-1	thrombospondin 1
TGF-β	transforming growth factor-β
UUO	unilateral ureteral obstruction

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Highlights

- TGF-β is a central mediator of fibrotic diseases
- Targeting the latent TGF-β activation mechanism is a selective approach to controlling TGF-β in disease
- The matricellular protein Thrombospondin-1 binds and activates latent TGF-β
- TSP-1 plays an important role in controlling TGF-β activation in fibrotic diseases, particularly in diabetic complications and in liver fibrosis
- Antagonists of the TSP-1/TGF-β axis represent a targeted approach to modulating TGF-β in disease

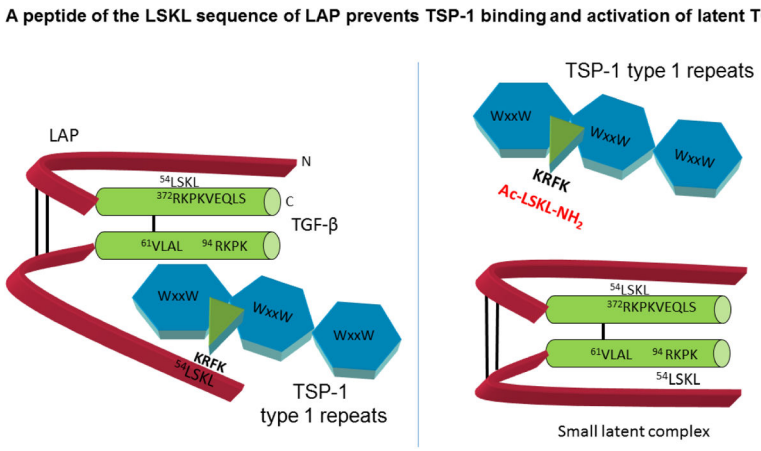

Figure 1. Latent TGF-β activation by TSP-1 and antagonism by peptides of the LAP LSKL sequence

TSP-1 binds to the TGF-β small latent complex comprised of the N-terminal latency associated peptide (LAP) and the C-terminal mature domain through its type 1 repeats. The C-terminus of the mature TGF-β homodimer binds to TGF-β signaling receptors. The small latent complex covalently binds to the fibrillin-like latent TGF-β binding protein (LTBP) at cysteine 33 of the LAP to form the large latent complex (not shown). Binding of the LSKL sequence at the N-terminus of the LAP to the RKPKVEQLS sequence in the receptor-binding region of the mature domain is necessary to confer latency [22, 25, 26]. The KRFK sequence of TSP1 located at the N-terminal portion of the second type 1 repeat of TSP-1 recognizes the LSKL sequence in the LAP and competitively disrupts LSKL-RKPKVEQLS binding to displace LAP from the mature domain. This results in access of the mature domain for TGF-β receptor binding and signaling as noted by changes in the secondary structure of the latent complex (data not shown). The tryptophan-rich motifs (WSxW) in each of the three type 1 repeats of TSP-1 also bind to the VLAL sequences in the mature domain and the LAP, although these interactions are not sufficient for activation. LSKL as a peptide can competitively block TSP-1 binding to the latent complex, thus preventing TSP-1 binding and activation of latent TGF-β. Using recombinant latent TGF-β1 and purified TSP-1, LSKL is an effective antagonist at picomolar concentrations (see supplemental data in [114].

Table 1

Disease models in which the TSP-1 antagonist LSKL impacts TGF-β activity and disease pathogenesis

Disease	Organ	In vivo model	TSP1 antagonist	Reference
Diabetes	Kidney	Mouse, Akita	LSKL	[28]
Diabetes/Hypertension	Heart	Rats, STZ with aortic coarctation	LSKL	[29]
Endomyocardial fibrosis	Heart	Id1/3 DKO adult mice	LSKL	[118]
Mesangial Proliferative glomerulonephritis	Kidney	Rats, Anti-Thy-1 antibody injury	LSKL,	[180]
Chronic Kidney Disease	Kidney	Rat, UUO	LSKL	[174]
Liver fibrosis	liver	Rat, DMN-induced	LSKL	[102]
Enhanced liver regeneration	liver	Mouse, partial hepatectomy	LSKL	[106]
Autoimmune	MS/Brain inflammation	EAE model of MS	LSKL, candesartan	[181]
Pulmonary arterial hypertension	Pulmonary artery	hypoxia-induced pulmonary arterial hypertension	LSKL	[116]
Arteriosclerosis	Carotid artery	Disturbed flow	LSKL	[117]
Subarachnoid fibrosis	brain	Rat, model of subarachnoid hemorrhage with hydrocephalus	LSKL	[182]
Multiple myeloma	Bone marrow	Heparanase expressing human myeloma/SCID mouse 5TGM1 syngeneic model	LSKL, SRI31277	[114]