Versican-thrombospondin-1 binding in vitro and colocalization in microfibrils induced by inflammation on vascular smooth muscle cells

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Summary

We identified a specific interaction between two secreted proteins, thrombospondin-1 and versican, that is induced during a toll-like receptor-3-dependent inflammatory response in vascular smooth muscle cells. Thrombospondin-1 binding to versican is modulated by divalent cations. This interaction is mediated by interaction of the G1 domain of versican with the N-module of thrombospondin-1 but only weakly with the corresponding N-terminal region of thrombospondin-2. The G1 domain of versican contains two Link modules, which are known to mediate TNFα-stimulated gene-6 protein binding to thrombospondin-1, and the related G1 domain of aggrecan is also recognized by thrombospondin-1. Therefore, thrombospondin-1 interacts with three members of the Link-containing hyaladherin family. On the surface of poly-I:C-stimulated vascular smooth muscle cells, versican organizes into fibrillar structures that contain elastin but are largely distinct from those formed by hyaluronan. Endogenous and exogenously added thrombospondin-1 incorporates into these structures. Binding of exogenous thrombospondin-1 to these structures, to purified versican and to its G1 domain is potently inhibited by heparin. At higher concentrations, exogenous thrombospondin-1 delays the poly-I:C induced formation of structures containing versican and elastin, suggesting that thrombospondin-1 negatively modulates this component of a vascular smooth muscle inflammatory response.

Key words: Matricellular proteins, Proteoglycan, Link modules, Vascular biology, Inflammation

Introduction

Vascular extracellular matrix (ECM) contains structural molecules that provide mechanical support and context-dependent signals necessary for differentiated vascular cell function (Brooke et al., 2003; Kelleher et al., 2004). These structural components of ECM including collagens, laminins, elastin, hyaluronan and proteoglycans are stably expressed over time. By contrast, some ECM components exhibit highly regulated or transient expression during development or in specific disease states. Several of the latter were termed matricellular proteins to distinguish them from the stably expressed structural ECM components (Bornstein, 1995). Matricellular proteins include thrombospondin-1 (TSP1), TSP2, tenascin-C and osteonectin/SPARC. TSP1 has a well-defined activity as an angiogenesis modulator but also regulates certain immune and inflammatory responses (reviewed in Adams and Lawler, 2004; Kuznetsova and Roberts, 2004). Its expression is induced during developmental remodeling, wound repair, in some tumor stroma, in rheumatoid synovium and in the adventitia of blood vessels from diabetic rats and atherosclerosis patients (Kuznetsova and Roberts, 2004; Stenina et al., 2003; Wight et al., 1985).

Proteoglycan components of ECM can similarly be categorized as primarily structural or regulatory molecules. Versican is a regulatory proteoglycan that is highly expressed by proliferating cells during tissue remodeling (reviewed in Kinsella et al., 2004; Wight, 2002). Roles for the elevated expression of versican have been proposed in several diseases including atherosclerosis, restenosis and cancer metastasis.

The versican gene encodes an alternatively spliced mRNA that gives rise to four versican isoforms (reviewed in Kinsella et al., 2004; Wight, 2002). Three of these include central GAG domains that are modified by chondroitin sulfate chains, but the V3 variant lacks these domains and is not a proteoglycan. All isoforms of versican contain the N-terminal G1 domain and the C-terminal G3 domain (Fig. 1B). The G1 region consists of an immunoglobulin fold followed by a contiguous pair of Link modules that mediate hyaluronan binding as is the case for most hyaladherins (Blundell et al., 2004; Day and Prestwich, 2002). The G3 domain of versican, which contains epidermal growth factor (EGF)-like, complement regulatory protein-like and C-type lectin-like modules, is a divalent cation-dependent ligand for β1 integrins (Wu et al., 2002). Versican also binds to other cell surface receptors including selectins, CD44 and P-selectin glycoprotein ligand-1 (Kawashima et al., 2000; Zheng et al., 2004). In the ECM, versican binds to other Link module-containing proteins in association with hyaluronan

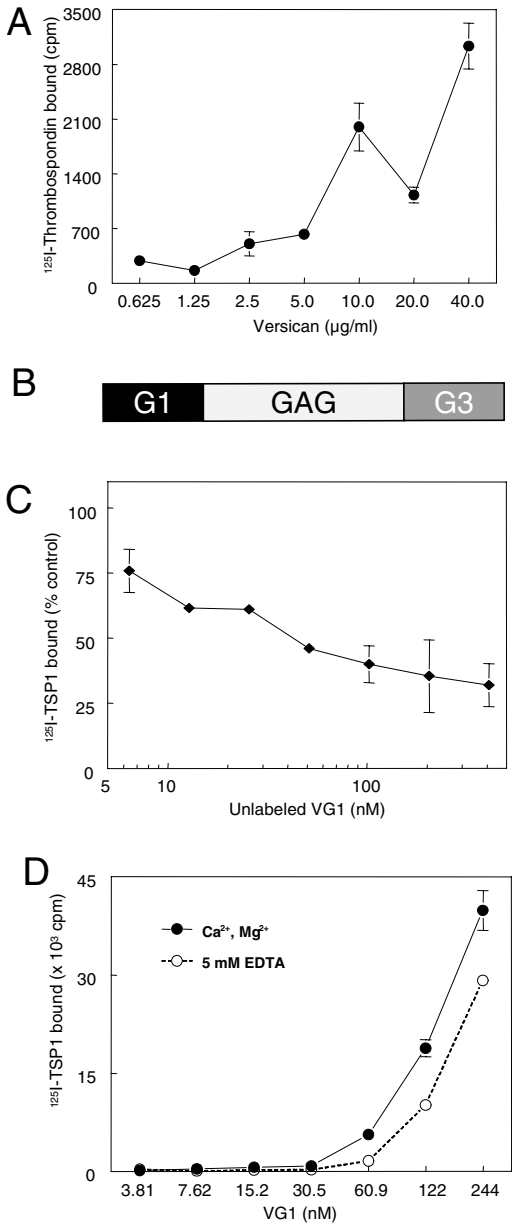

Fig. 1. TSP1 binding to versican. (A) TSP1 binding to immobilized full-length versican. Wells were coated using the indicated concentrations of versican, blocked with 3% BSA and incubated with 0.5 μg ml^{-1} (1.1 nM) of [^{125}I]-TSP1 in buffers containing Ca^{2+} and Mg^{2+} for 3 hours at 37°C. Background values were subtracted from each data point (211.2±19.1). (B) Versican core protein contains an amino terminal hyaluronan-binding region containing an immunoglobulin and 2 Link protein modules (G1), a variably spliced glycosaminoglycan-attachment domain (GAG) and a C-terminal domain (G3) containing two epidermal growth factor-like repeat, a C-type lectin-like module and a complement regulatory protein-like module. (C) Binding of [^{125}I]-TSP1 to wells coated using 1 μg per well of versican was determined in the presence of the indicated concentrations of VG1. Results are normalized to net binding measured in the absence of VG1 (748.8±24.2 cpm). (D) Binding of [^{125}I]-TSP1 to wells coated with the indicated concentrations of VG1 in buffers containing Ca^{2+} and Mg^{2+} (●) or 5 mM EDTA (○) for 3 hours at 37°C. Background values were subtracted from each data point (137.3±13.2 and 87.2±9.6, respectively). All data are mean ± s.e.m. for quadruplicate determinations.

(Matsumoto et al., 2003; Seyfried et al., 2005) and to some chemokines (Hirose et al., 2001).

TSP1 typically binds to heparan sulfate proteoglycans through their glycosaminoglycan chains, mediated primarily by the high affinity heparin binding site in the N module of TSP1 (Dixit et al., 1984; Yu et al., 2000). However, TSP1 also interacts with the core protein of at least one proteoglycan, decorin (Winnemoller et al., 1992). This interaction inhibits fibroblast adhesion to the N-domain of TSP1 (Merle et al., 1997), suggesting that the N module of TSP1 mediates protein-protein interactions with this proteoglycan.

We recently found that TSP1 binds with high affinity to the Link module of tumor necrosis factor-induced protein-6 (TSG-6) (Kuznetsova et al., 2005). This interaction is mediated by the N module of TSP1. Link modules occur in a number of proteoglycans including versican and aggrecan. The conservation of primary sequences and 3D folding among the Link modules of hyaladherins (Blundell et al., 2005) suggested that TSP1 binding could be conserved among other members of the hyaladherin family. We report here that the G1 domains of versican and aggrecan containing Link modules are also recognized by TSP1 via its N-terminal region. Based on similar reports of TSP1 and versican over-expression during inflammatory responses involving vascular smooth muscle cells (VSMC) (Kinsella et al., 2004; Riessen et al., 1998; Wight and Merrilees, 2004), we hypothesized that interaction of versican with TSP1 would occur in this context. We show here that versican and TSP1 are coordinately upregulated in the ECM of VSMC during a toll-like receptor 3 (TLR3)-induced inflammatory response and organize into fibrillar structures that also contain elastin. Furthermore, we demonstrate that TSP1 at high concentrations is a negative regulator of this inflammatory process.

Results

Thrombospondin-1 binds versican and aggrecan G1 domains

A solid phase assay using [^{125}I]-TSP1 and immobilized versican was used to assess binding of TSP1 to native versican (Fig. 1A). Dose-dependent binding was observed at 37°C, pH 7.3 in the presence of physiological salt concentrations and divalent cations. This binding could be mediated by TSP1 binding either to a glycosaminoglycan on the GAG domain of versican or to the core protein (Fig. 1B). As shown in Fig. 1C, recombinant G1 domain of versican (VG1), which contains N-linked glycosylation but is free of glycosaminoglycans (Seyfried et al., 2005), inhibits TSP1 binding to native versican. Dose-dependent binding of TSP1 was also observed to immobilized VG1 (Fig. 1D), indicating a direct protein-protein interaction of TSP1 with this domain. Similar to the interaction we recently reported between TSP1 and TSG-6 (Kuznetsova et al., 2005), this binding was sensitive to divalent cations and partially inhibited in the presence of EDTA.

Competitive displacement of [^{125}I]-TSP1 by unlabeled TSP1 was used to confirm specific binding to immobilized VG1 (Fig. 2A). Saturable binding was observed in the presence or absence of divalent cations. These data could be fit to a one-site model. TSP1 bound to immobilized VG1 with a K_a of 3.5×10^8 M^{-1} in the presence of divalent cations and 2.2×10^9 M^{-1} in the absence of cations. A similar enhancement of TSP1 binding affinity for the Link module of TSG-6 was observed

in the absence of cations, which may result from a Ca^{2+}-mediated conformational change in TSP1 (Kuznetsova et al., 2005; Rodrigues et al., 2001).

Although the primary sequences of Link modules are quite divergent, with only 30-40% sequence identity (Blundell et al., 2005), these data suggested that TSP1 binding might be a general property of the Link module superfamily. To test this hypothesis, we used a reverse assay in which TSP1 was immobilized and incubated with biotinylated G1 domains from versican or aggrecan and detected binding using [^{125}I]-streptavidin (Fig. 2). Both VG1 and AG1 bound in a dose-dependent manner to immobilized TSP1. Consistent with a previous report that Link_TSG6 interacts with the G1-domain of aggrecan purified from porcine laryngeal cartilage (Parkar et al., 1998), human recombinant AG1 also bound in a dose dependent manner to immobilized TSG-6 (Fig. 2C). This confirms the functional activity of the biotinylated AG1 and is the first demonstration of an interaction between human TSG-6 and human aggrecan. Biotinylated VG1 also bound to immobilized Link_TSG6 (Fig. 2B), indicating that these proteins may also interact through their Link modules.

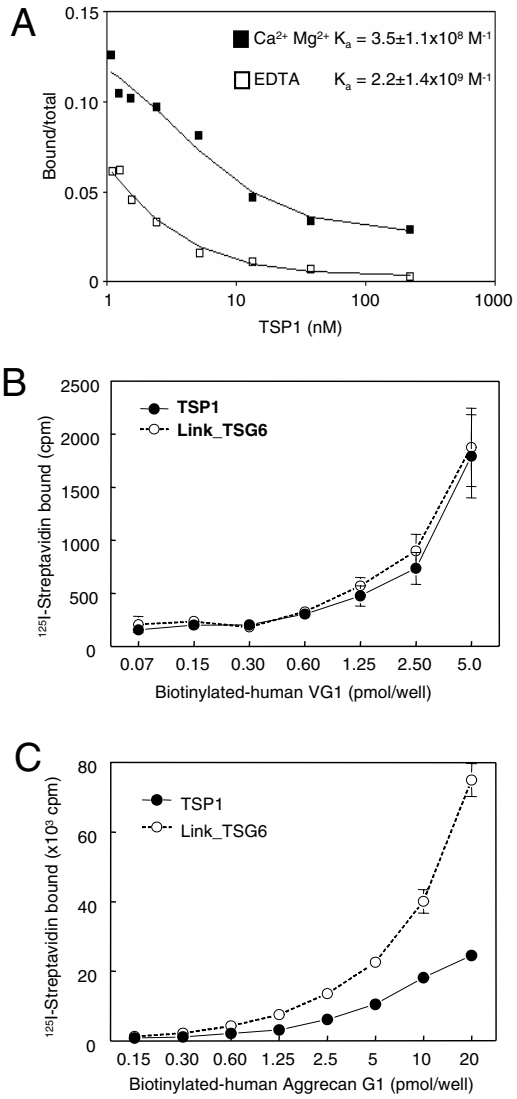

Fig. 2. Binding of versican and aggrecan G1 domains to immobilized TSP1. (A) Self displacement of TSP1 binding to versican G1 domain. Wells coated with 0.26 μg per well of VG1 were incubated at 37°C with 0.25 μg ml^{-1} [^{125}I]-TSP1 in the presence of unlabelled TSP1 at 0.25-100 μg ml^{-1} in buffers containing Ca^{2+} and Mg^{2+} (■) or 5 mM EDTA (□). The curves represent the best fit displacement curves calculated for a single site model using the program LIGAND. (B) Wells were coated using 0.5 μg per well TSP1, blocked with 3% BSA for 1 hour and incubated with the indicated amounts of biotinylated G1 domains of versican (B) or aggrecan (C) at 37°C for 3 hours in a final volume of 50 μl of DPBS with Ca^{2+} and Mg^{2+} containing 0.5% BSA and 0.1 mM phenylmethylsulfonyl fluoride. After washing to remove unbound proteins, the wells were incubated with 1 μg ml^{-1} [^{125}I]-streptavidin for 1 hour at room temperature, washed and the bound radioactivity quantified. Background values were subtracted from each data point (1150.8±99.2).

Interaction of TSP1 with VG1 is mediated by the N-terminal region of TSP1, based on competition by several recombinant regions of TSP1 (Fig. 3A). A trimeric N-terminal construct containing the N module, oligomerization site and vWC modules (Fig. 3B) was equipotent with full length TSP1 on a molar basis. All constructs lacking the N module failed to achieve 50% inhibition over the dose range tested. Among these, the CP123 construct also contains the vWC module of TSP1 which is present in the active construct NoC1, suggesting that the VG1 binding site in TSP1 is located in its N module, as was inferred for TSP1 binding to the link domain of TSG-6 (Kuznetsova et al., 2005). This mechanism was directly demonstrated by the activity of recombinant monomeric N module of TSP1 to inhibit [^{125}I]-TSP1 binding to immobilized VG1 (Fig. 3C).

We recently found that the ability to recognize the Link module of TSG-6 is conserved in TSP2, although TSP2 was approximately fourfold less active than TSP1 (Kuznetsova et al., 2005). A recombinant trimeric N-terminal region of TSP2 (NoC2) also competed with [^{125}I]-TSP1 for binding to immobilized VG1 (Fig. 3C). However, its IC_{50} was more than tenfold higher than for the corresponding region of TSP1 (NoC1). Therefore, versican selectively interacts with TSP1.

To confirm the role of the N-terminal domains of TSP1 in binding to native versican, we examined direct binding of [^{125}I]-NoC1 to immobilized versican (Fig. 4A) and VG1 (Fig. 4B). Binding of NoC1 to versican was divalent cation-dependent and was enhanced in the presence of 5 mM EDTA. Unlike native versican, NoC1 binding to VG1 was identical in the presence and absence of divalent cations, indicating that divalent cations do not directly influence this protein-protein interaction. TSP1 is known to have multiple divalent cation binding sites in its C-terminal domains that modulate its conformation (Hannah et al., 2003; Kvansakul et al., 2004; Lawler et al., 1985) and the conformation of intact versican may similarly be altered by divalent cation binding to modulate its accessibility to TSP1 binding.

The N module of TSP1 contains a high affinity heparin binding site and its occupancy by heparin inhibits TSP1 binding to TSG-6 (Kuznetsova et al., 2005). Heparin similarly inhibited binding of [^{125}I]-TSP1 to both recombinant VG1 and native versican (Fig. 4C). The similar IC_{50} values for binding to VG1 and versican are further evidence that both interactions are mediated by the N modules of TSP1.

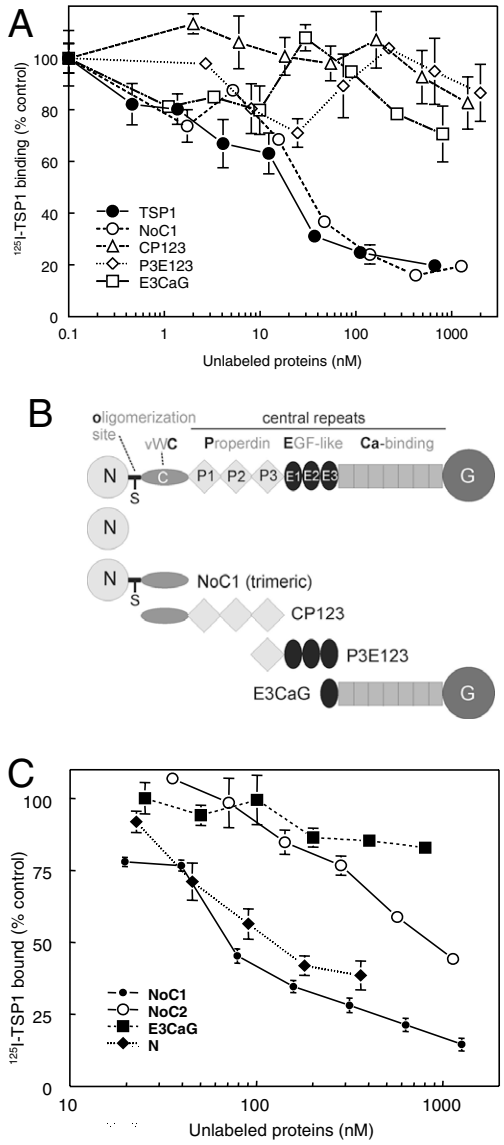

Fig. 3. Mapping of the versican-binding domain in TSP1. (A) [^{125}I]-TSP1 binding to immobilized VG1 was determined in the presence of divalent cations and the indicated concentrations of full length TSP1 (●), NoC1 (○), CP123 (△), P3E123 (◇), or E3CaG (□). (B) A structural model of TSP1. (C) [^{125}I]-TSP1 binding to immobilized VG1 was determined in the presence of divalent cations and the indicated concentrations of NoC1 (●), NoC2 (○), E3CaG (■), or N (◆).

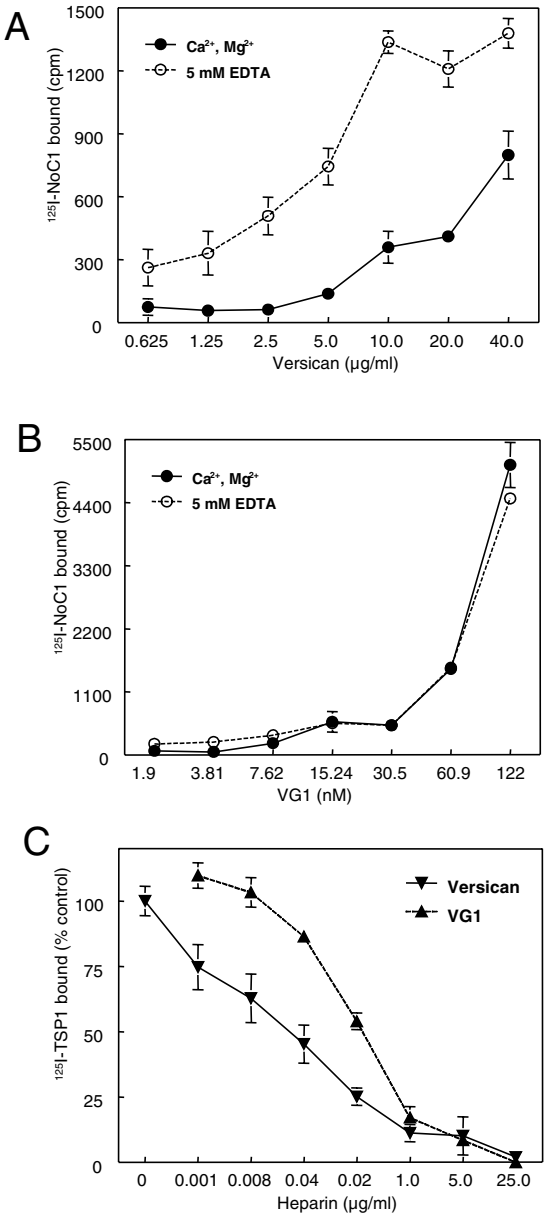

Fig. 4. NoC1 binding to versican. Binding of [^{125}I]-NoC1 to wells coated with the indicated concentrations of full-length versican (A) or VG1 (B) in buffers containing Ca^{2+} and Mg^{2+} (●) or 5 mM EDTA (○) for 3 hours at 37°C. Background values were subtracted from each data point (365.8±28.7 and 286.0±21.3). (C) Binding of [^{125}I]-TSP1 to wells coated using 1 μg per well of versican (▼) or 0.3 μg per well of VG1 (▲) was determined in the presence of the indicated concentrations of heparin. Binding was normalized to that determined in the absence of heparin (748.8±24.2 and 12658.8±117.4 cpm, respectively). All data are mean ± s.e.m. for quadruplicate determinations.

TSP1 and versican are induced and co-localize during an inflammatory response in HASMC

TSP1 and versican have been independently reported to be up-regulated during inflammatory responses of VSMC that are associated with atherosclerosis (Riessen et al., 1998; Wight and Merrilees, 2004), but their potential co-localization or interaction under these conditions has not been considered. Based on our biochemical data, we determined whether endogenous TSP1 co-localized with versican in cultured HASMC. To evoke an inflammatory response in vitro, we exposed HASMC to the TLR3 ligand poly I:C, which induces an endoplasmic reticulum (ER) stress response in these cells (Majors et al., 2003). By immunofluorescence, extracellular TSP1 was barely detectable in unstimulated cells (Fig. 5A left panel), but staining of permeabilized cells revealed punctate perinuclear staining (Fig. 5B left panel). Versican showed similar perinuclear staining in unstimulated HASMC that partially overlapped with TSP1 (Fig. 5B left). After 4 hours of

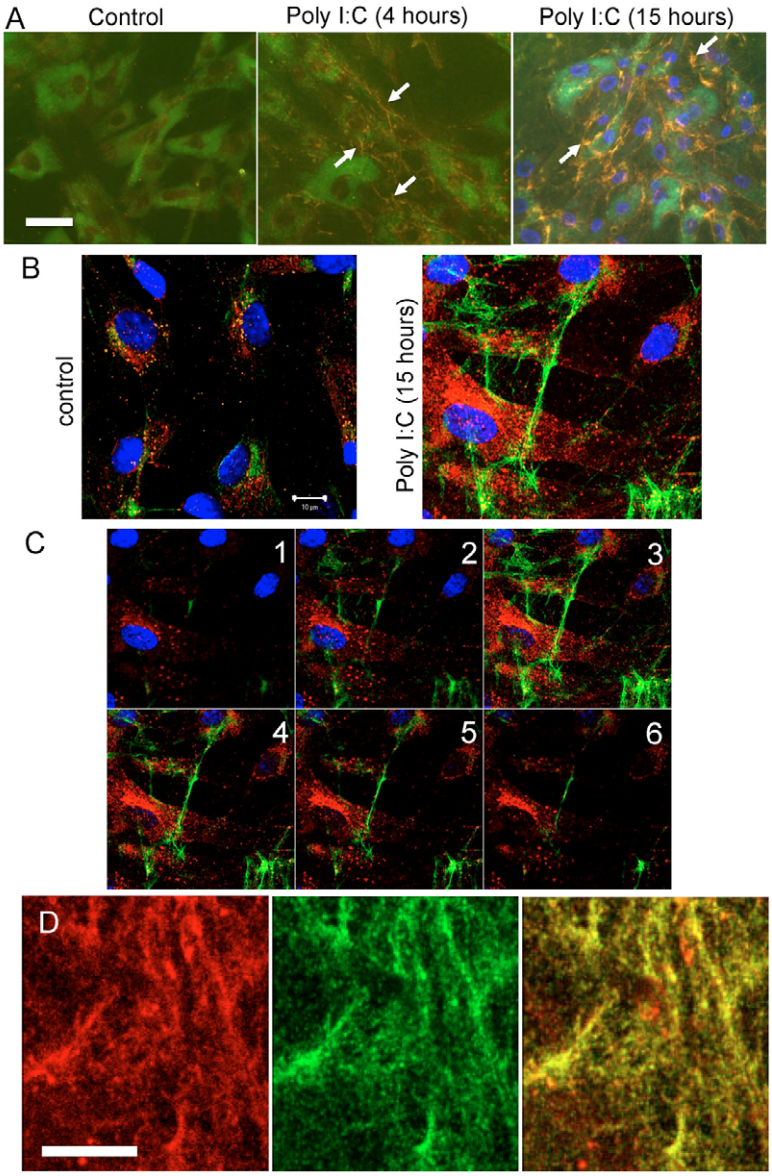

Fig. 5. Induction of extracellular matrix TSP1 and versican by a TLR3 agonist. (A) HASMC were incubated in control medium or medium containing 5 μg ml^{-1} poly I:C for 4 hours or 15 hours, followed by fixation and double immunofluorescent staining without permeabilization using antibodies to versican (green) and to TSP1 (red). Nuclei were counterstained with Hoechst 33258 (blue). Note that co-localization of versican and TSP1 appears as yellow close to the cell surface after treatment of HASMC with poly I:C in a time-dependent manner. Bars, 50 μm. Results are representative of at least three independent experiments. (B,C) Confocal images showing extracellular localization of TSP1 and versican after stimulation with poly I:C. HASMC were cultured without treatment (B, left) or treated with 5 μg ml^{-1} poly I:C for 15 hours (B, right and C) and cells were fixed, permeabilized and double stained for versican (green) and TSP1 (red). Stack of images (C, 1-6) through the Z-plane from inside (1) of the cells to the cell surface was generated using confocal microscopy. Nuclei were counterstained with DAPI (blue). Bar, 10 μm. (D) Confocal 3D reconstruction of 18 hour poly I:C-stimulated HASMC stained without permeabilization for versican (green) and TSP1 (red). Bar, 10 μm.

treatment with poly I:C, coincident fibrillar staining of TSP1 and versican appeared in the matrix (yellow areas indicated by arrows in Fig. 5A) and became more prominent after 15 hours. TSP1 initially associated with thin strands that after overnight activation coalesced into the thicker fibrillar structures. Extracellular versican and TSP1 showed extensive colocalization in these structures when visualized at higher magnification on nonpermeabilized cells in confocal 3D reconstructions (Fig. 5D). Confocal XY sections of permeabilized cells verified that most of the fibrillar structures induced following stimulation with poly I:C localized above the plane of the nuclei and concentrated to the extracellular space between adjacent cells (Fig. 5B,C).

A previous study using colonic mucosal smooth muscle cells did not find increased versican expression following poly I:C stimulation but reported induction of cable-like structures containing hyaluronan (de la Motte et al., 2003). Because versican binds to hyaluronan (Matsumoto et al., 2003; Seyfried et al., 2005) and is also found in hyaluronan cables induced by ER stress (Majors et al., 2003), we examined whether the structures induced in HASMC that contain TSP1 and versican co-localized with hyaluronan. Although hyaluronan in the matrix was strongly induced following poly I:C stimulation (Fig. 6A and data not shown), only minimal overlap was observed between structures containing versican and those containing hyaluronan. Notably, some cells in response to poly I:C elaborate an hyaluronan-rich matrix containing relatively little versican (indicated by *), whereas other cells elaborate a versican-rich matrix containing little hyaluronan (indicated by arrows).

TSP1 association with elastin microfibrils

Versican is also known to associate with elastin-containing fibrils through its interaction with fibrillin-1 (Isogai et al., 2002). Tropoelastin synthesis by rat ASMC is induced following over expression of versican V3 in vitro and in vivo (Merrilees et al., 2002). To determine whether extracellular TSP1 induced by poly I:C was associated with elastin-containing fibrils, we performed two-color immunostaining for extracellular elastin and TSP1 in nonpermeabilized cells (Fig. 6B). Elastin staining was increased following poly I:C stimulation for 4 hours and overlapped extensively with the induced TSP1 immunoreactivity (yellow areas).

Exogenous TSP1 added into the culture medium further enhanced binding to these structures formed by HASMC stimulated with poly I:C relative to unstimulated cells (Fig. 7). This suggests that endogenous TSP1 is not sufficient to saturate the available TSP1 binding sites in the fibrillar structures. Exogenous TSP1 binding to pre-activated HASMC was maximal within 1.5 hour, but following prolonged incubation (15 hours) the extracellular labeling disappeared and was replaced by punctate TSP1 staining that may indicate internalization of these complexes, as reported in other cell

types (Godyna et al., 1995; Gonias et al., 2004). To confirm internalization, exogenous TSP1 was added into the culture medium simultaneously with poly I:C for 4 hours. By confocal imaging of permeabilized and non-permeabilized cells in the plane of the nucleus (Fig. 7B left panels) and a plane that included the cell surface and subsurface actin cytoskeleton (Fig. 7B right panels) TSP1 was localized to small punctate structures inside the cells and on the cell surface.

As was shown for endogenous TSP1, the bound exogenous TSP1 partially colocalized with versican on poly I:C pre-

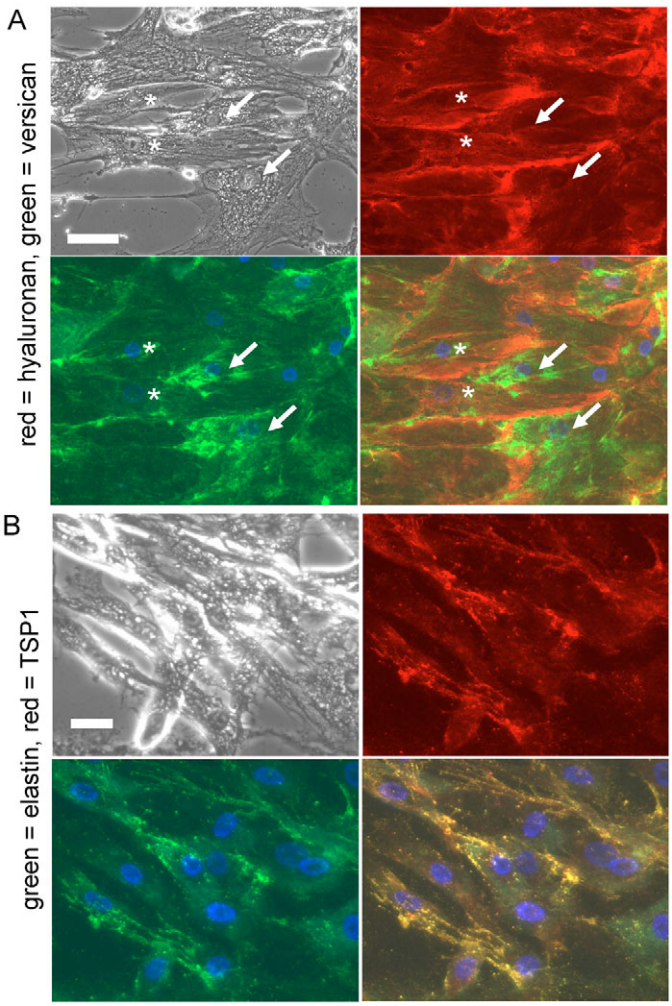

Fig. 6. Distinct localization of extracellular hyaluronan and proteins induced by poly I:C. (A) Versican-rich matrix generated by inflammation is mostly distinct from the stress-induced hyaluronan-rich matrix. Image of versican (green, bottom left quarter) and hyaluronan (red, top right) are shown together with phase contrast image (top left) and merge (bottom right). Only limited co-localization of versican and hyaluronan was observed after stimulation with 5 μg ml^{-1} poly I:C for 17 hours. (B) Inflammation induces increased accumulation and co-localization of TSP1 with elastin. HASMC were cultured with 5 μg ml^{-1} poly I:C for 4 hours before being processed for immunofluorescence staining. Note that the anti-elastin antibody (green) binds to many of the same structures that are labeled with the anti-TSP1 antibody (red). Nuclei were counterstained with Hoechst 33258 (blue). Bars, 50 μm (A); 20 μm (B).

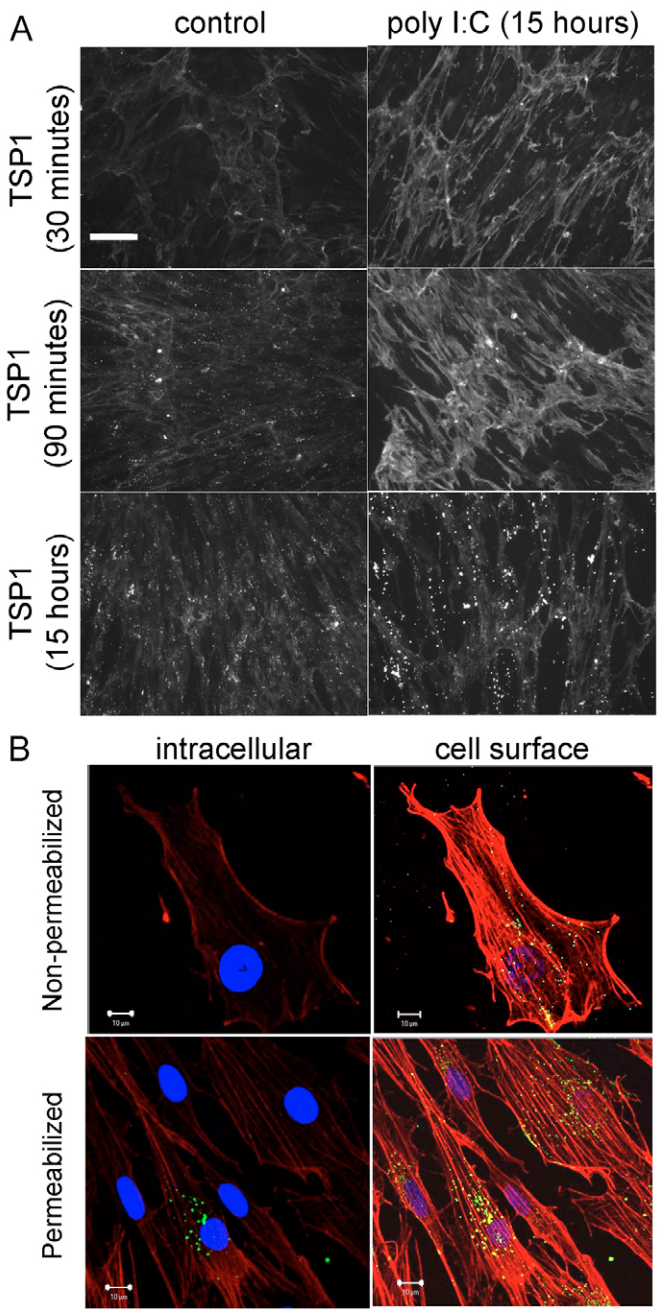

Fig. 7. Inflammation-induced binding and turnover of exogenous TSP1. (A) HASMC were cultured for 15 h in either control (left panels) or 5 μg ml^{-1} poly I:C containing medium (right panels). TSP1 (10 μg ml^{-1}) was added at the indicated time points before completion of the 15 hour incubation period. Immunofluorescent staining of nonpermeabilized cells shows that TSP1 binding to the extracellular matrix is induced by poly I:C in a time dependent manner. However, prolonged incubation with soluble TSP1 in the presence of poly I:C shows reorganization of matrix-associated TSP1 into punctate structures. (B) Confocal microscopy of HASMC, followed by stimulation with 5 μg ml^{-1} poly I:C in the presence of TSP1 (10 μg ml^{-1}) at 4 hours. Permeabilized or non-permeabilized cells were fixed and stained for TSP1 (green), nuclei (blue) and F-actin (red). Representative confocal images showing that TSP1 containing punctate structures have both an intracellular (bottom, left) and an extracellular distribution (upper right panel). Bars, 50 μm (A); 10 μm (B).

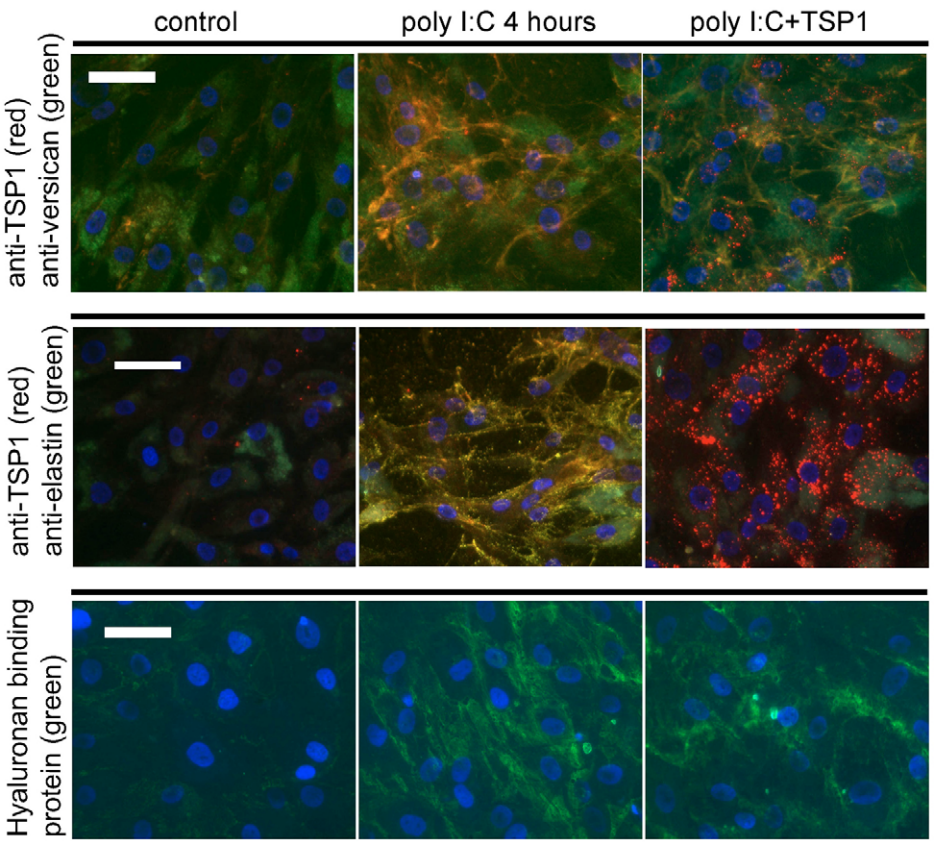

Fig. 8. High concentrations of exogenous TSP1 inhibit inflammation-induced extracellular deposition of versican and elastin. HASMC were cultured without (control) or with 5 μg ml^{-1} poly I:C for 4 hours in the presence or absence of 20 μg ml^{-1} TSP1. Cells were subsequently fixed and double stained as indicated in the Materials and Methods. Exogenous TSP1 diminished versican and elastin accumulation, but not hyaluronan, as seen by double immunofluorescence staining for TSP1 (red), for versican, elastin or hyaluronan (green) and nuclei (blue). Bars, 50 μm.

activated HASMC. Furthermore, these structures were distinct from hyaluronan, TSG-6, or CD44 immunoreactivity (results not shown).

TSP1 delays fibril induction by poly-I:C

Based on the results in Fig. 7, we considered that exogenous TSP1 may limit formation of microfibrils containing versican and elastin by stimulating their internalization and catabolism. Consistent with this hypothesis, adding higher concentrations of exogenous TSP1 inhibited the fibrillar elastin and versican staining induced by poly I:C after 4 hours (Fig. 8). TSP1 staining in the matrix was also lost and replaced by prominent staining of intracellular granules. By contrast, adding exogenous TSP1 only moderately inhibited hyaluronan induction in the matrix of HASMC stimulated with poly I:C for 4 hours (Fig. 8, lower panel).

Consistent with the activity of heparin to inhibit TSP1 binding to the Link domain of versican, heparin inhibited binding of exogenous TSP1 to poly I:C-activated HASMC (Fig. 9). Poly I:C-induced fibrillar TSP1-staining was significantly reduced in the presence of 1 μg ml^{-1} heparin and essentially blocked by 100 μg ml^{-1} heparin. Addition of 100 μg ml^{-1} heparin to unstimulated cells did not significantly alter TSP1 staining.

TLR3 stimulation alters TSP1 localization

The observed increases in TSP1, versican and elastin immunoreactivity could indicate increased synthesis or secretion of these proteins or the induction by poly I:C of a structure to which these secreted proteins bind. Real time-PCR was used to quantify mRNA levels for elastin, versican and

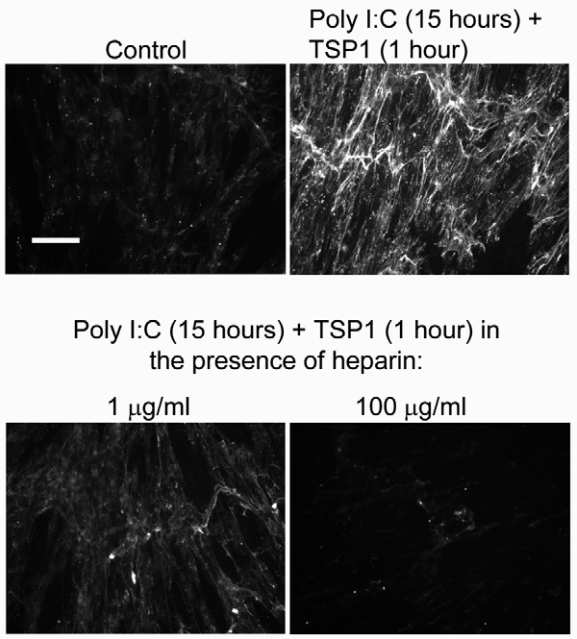

Fig. 9. Heparin inhibits TLR3-induced deposition of exogenous TSP1. HASMC were cultured in standard medium (control) or in the presence of 5 μg ml^{-1} poly I:C for 15 hours, followed by an additional 1 hour incubation in culture medium containing 10 μg ml^{-1} TSP1 with or without the indicated concentrations of heparin. Staining with antibody against TSP1 showed that binding of exogenous protein to the structures generated by cells following poly I:C treatment was inhibited by heparin in a dose-dependent manner. Bar, 50 μm.

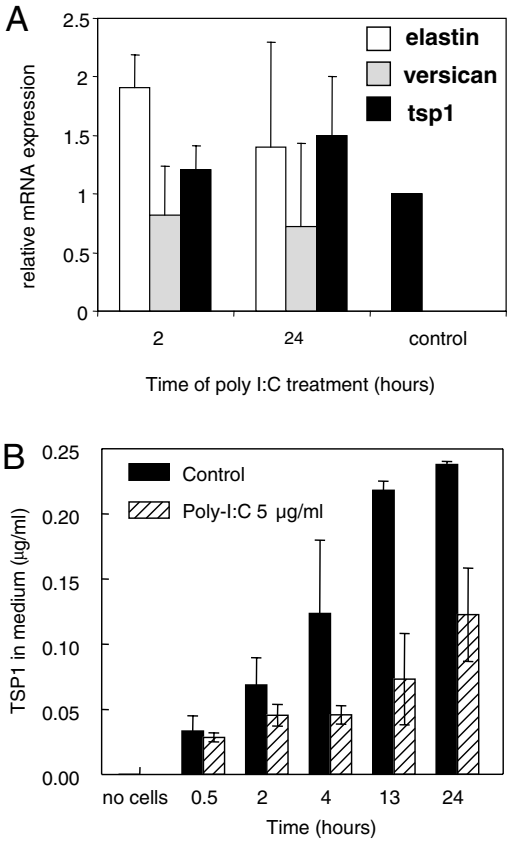

Fig. 10. Effects of poly I:C on matrix protein synthesis and secretion. (A) Real time PCR. HASMCs were treated with or without 5 μg ml^{-1} poly I:C for 2 or 24 hours and relative mRNA expression levels using Taqman probes for elastin, versican and TSP1 were quantified using the comparative C_t method with HPRT as the internal standard. Expression data is normalized to that of untreated cells. The graph represents 2 separate experiments, each run in duplicate. All data are mean ± s.d. for quadruplicate determinations. (B) Effect of poly I:C treatment on accumulation of TSP1 in the medium of HASMC at the indicated times following transfer to serum free medium (closed bar) or the same medium containing 5 μg ml^{-1} poly I:C.

TSP1 (Fig. 10A). Elastin mRNA was moderately induced at 2 hours following addition of poly I:C, but TSP1 and versican mRNA levels were not significantly altered by poly I:C treatment after 2 or 24 hours. By contrast, secreted TSP1 protein levels were significantly reduced in poly I:C-treated VSMC at 4-24 hours (Fig. 10B). This indicates that the increased extracellular deposition of TSP1 in the matrix generated by poly I:C treated cells is recruited from the basally secreted TSP1 rather than resulting from increased synthesis or secretion.

Discussion

Extending our previous observation that TSP1 binds with high affinity to the Link module of TSG-6 (Kuznetsova et al., 2005), we now show that TSP1 recognizes the Link-containing domains of two additional hyaladherins, aggrecan and versican. Binding to VG1 is mediated by the N-terminal region of TSP1 and shows a similar divalent cation dependence as noted previously for TSG-6 (Kuznetsova et al., 2005). The interaction of TSP1 with versican appears to be relatively specific for TSP1 in that the N-terminal region of TSP2 was 10-fold less active as an inhibitor of TSP1 binding to VG1. Fibrillar structures containing TSP1, elastin and versican are induced during an inflammatory response in HASMC. Based on its ability to interact with TSP1 and fibrillin-1 through different domains (Isogai et al., 2002), versican may be the bridging molecule that mediates TSP1 binding to microfibrils (Fig. 11). Although these structures are clearly induced during TLR3-mediated inflammation, the molecular events that trigger their assembly remain unclear. TSP1 appears to be derived from redirecting its constitutive secretion by the cells rather than increased biosynthesis. Versican mRNA is also unperturbed, whereas elastin mRNA is moderately induced.

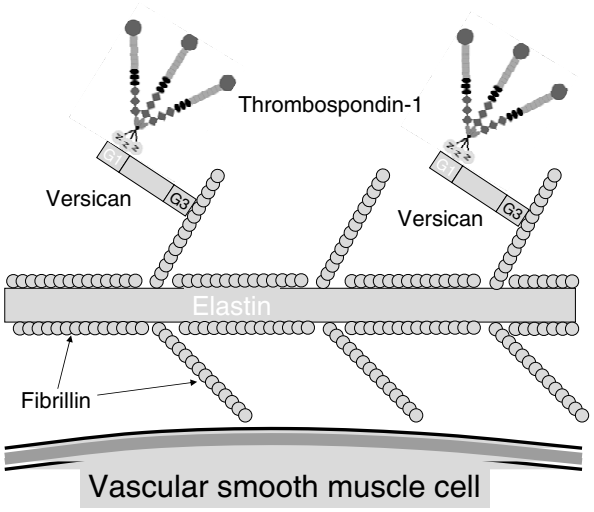

Fig. 11. Model for inflammation-induced matrix complexes on VSMC.

Our results are consistent with previous reports of fibrillar structures in the subendothelial matrix of arteries that contain either TSP1 or versican in association with microfibrils (Fauvel-Lafeve, 1999; Fauvel-Lafeve and Legrand, 1988; Isogai et al., 2002). An antiserum specific for elastin-associated microfibrils in the arterial wall was shown to recognize TSP1 (Fauvel-Lafeve et al., 1988). Subsequent studies extended this association with TSP1 to microfibrils associated with elastin or basement membranes in other tissues (reviewed in Fauvel-Lafeve, 1999). Independently, versican was shown to covalently associate with fibrillin-1 in microfibrils via its G3 domain (Isogai et al., 2002).

Expression of the versican variant V3 induced elastic fiber formation by long term cultures of aortic smooth muscle cells and induced elastic fibers in the neointima following balloon catheterization of rat carotid arteries (Merrilees et al., 2002). Furthermore, versican V3 expression rescued the impaired elastic fiber formation in fibroblasts from Costello disease and Hurler disease patients (Hinek et al., 2004). Because TSP1 binds to G1 and fibrillin-1 binds to the G3 domain of versican, we propose that the colocalization of TSP1 with versican elaborated by VSMC during an inflammatory response involves binding of TSP1 to versican, which is anchored to fibrillin-1 (Fig. 12). Further research is needed to confirm this

complex and to determine how TSP1 binding modifies the properties of these microfibrils.

The potential biological significance of TSP1-versican binding in atherosclerosis should be considered in the context that a TSP1 antibody reduced neointima formation in balloon-injured rat carotid arteries (Chen et al., 1999). A single nucleotide polymorphism in the human *THBS1* gene, which alters the conformation of the Ca^{2+}-binding repeats of TSP1 (Hannah et al., 2003), is associated with premature coronary artery disease (Stenina et al., 2004). These data suggest that TSP1 in the adventitial ECM is limiting for neointimal formation and its Ca^{2+}-dependent conformation, which also regulates both TSG-6 and versican binding, is associated with coronary artery disease. The conformation of TSP1, therefore, may influence neointima formation through altering its interactions with versican and potentially other ECM components. However, TSP1 also interacts with several receptors on VSMC and thereby has direct effects on HASMC proliferation and migration (Isenberg et al., 2005; Lee et al., 2003; Lymn et al., 2002; Yabkowitz et al., 1993). Thus, TSP1 bound to versican on microfibrils may alter VSMC responses by providing prolonged stimulation of TSP1 receptors on these VSMC.

TSP1 and versican have been individually implicated in tumor progression, so the knowledge that they are binding partners may further our understanding of their function in cancer. Stromal induction of TSP1 is characteristic of the inflammatory responses induced by several cancers and TSP1 has both pro- and anti-inflammatory activities toward specific immune cell types (Kuznetsova and Roberts, 2004). Our data indicates that TSP1 may have an additional anti-inflammatory activity through limiting inflammatory responses in VSMC. Our data suggests a role of TSP1 in internalization of microfibrils containing versican, but direct effects of TSP1 on VSMC signaling have been described (Isenberg et al., 2005; Lymn et al., 2002) and should also be considered in interpreting our results.

Stromal expression of TSP1 is induced in some cancers and in some cases limits tumor progression (Fontana et al., 2005; Lawler and Detmar, 2004). Coordinated up-regulation of TSP1 and versican was reported in stromal cells of human breast carcinomas (Brown et al., 1999). Thus, binding of TSP1 to versican may also be relevant to the stromal inflammatory responses that are critical to progression of some cancers (reviewed in Bhowmick et al., 2004).

Materials and Methods

Protein purification

TSP1 was purified from human platelets obtained from the National Institutes of Health Department of Transfusion Medicine under a protocol approved by the NIH Institutional Review Board and stored at –70°C in 0.02 M Tris, pH 7.6, 0.15 M NaCl, 0.1 mM $CaCl_2$, 20% w/v sucrose (Roberts et al., 1994). Monomeric and trimeric recombinant regions of TSP1 and TSP2 expressed in insect cells were prepared as described (Anilkumar et al., 2002; Misenheimer et al., 2000). Versican was purified from bovine aorta using methods described in (Olin et al., 1999; Sandy et al., 2001).

Monomeric recombinant N module containing residues 1 to 250 of mature TSP1 was expressed using the Invitrogen FastBac1 baculovirus vector p6SXTE containing a full length TSP1 cDNA was amplified by PCR using forward primer CTCCGGTACACACAGGATCCCTGCTG and reverse primer TATGAATTC-ATGATGATGATGATGATGGCCGGCGGCTTGCAAGTCCTTTG. The resulting PCR product containing the TSP1 leader sequence and AHHHHHH appended on the C-terminus was ligated into the pCR-Blunt vector (Invitrogen). After verifying by sequencing, the insert was excised by *Eco*RI digestion and ligated into the pFastBac vector. Generation of the recombinant baculovirus and protein expression were done according to the Invitrogen Bac-to-Bac procedure using Sf-21 insect cells. Conditioned medium was dialyzed into 20 mM Tris, 350 mM NaCl, 20 mM imidazole, pH 7.5 and then passed through a Ni-NTA agarose column (Sigma). Bound N module was eluted from the column with 20 mM Tris, 350 mM NaCl, 300 mM imidazole, pH 7.5. The isolated protein was dialyzed into 20 mM Tris, 20 mM $NaHCO_3$, 350 mM NaCl, pH 8.5 and stored at –70°C.

TSP proteins/domains and streptavidin were labeled with [^{125}I] using Iodogen (Pierce, Rockford, IL) as described previously (Guo et al., 1992). G1 domains of versican (VG1) and aggrecan (AG1) were prepared as described (Seyfried et al., 2005). These were biotinylated essentially as described previously for cartilage link protein (Seyfried et al., 2005). Briefly, VG1 or AG1 (2.5 ml at ~10-20 μg ml^{-1}), purified by ion exchange chromatography (Seyfried et al., 2005), were incubated with 162 μl of 5 mg ml^{-1} medical grade high molecular weight hyaluronan (Genzyme; ~1.2 MDa) for 1 hour at room temperature. This was followed by addition of 18 μl of ovine testicular hyaluronidase (7000 U ml^{-1} in 20 mM MES, 5 mM EDTA, pH 6.5) and incubation for 1 hour at 37°C. To this was added 2.5 ml 34 μg ml^{-1} NHS-LC biotin (Pierce) in 100 mM $NaHCO_3$, pH 8.5 and mixed by rotation for 1 hour at room temperature. The biotinylated proteins were then purified by reverse-phase HPLC as described before for the unmodified AG1 and VG1 and quantified by amino acid analysis (Seyfried et al., 2005).

Chemicals and antibodies

Heparin sodium salt from porcine intestinal mucosa (M_r ~12,000) was purchased from Eli Lilly, Indianapolis, IN. Hanks' Balanced Salt Solution (HBSS) containing calcium and magnesium without phenol red and Dulbecco's PBS with or without divalent cations were from Invitrogen. Poly-I:C was from Invivogen. Paraformaldehyde and bovine serum albumin (BSA) were from Sigma-Aldrich.

Biotinylated hyaluronic acid binding protein (HABP) and mouse anti-human large proteoglycan (versican) antibody (2-B-1) were from Seikagaku Corporation. Mouse anti-TSP1 antibody (Ab-4, clone A6.1) purified or biotin-labeled were from NeoMarkers. A rabbit polyclonal TSP1 antibody R187 was described previously (Isenberg et al., 2005). Mouse anti-elastin antibody was a gift of Dr Robert Mecham (Washington University, St Louis). Bodipy-FL-goat anti-mouse IgG (H+L) conjugate, Alexa Fluor 488 F(ab′)$_2$ fragment of goat anti-mouse IgG (H+L), Alexa Fluor 568 F(ab′)$_2$ fragment of goat anti-rabbit IgG (H+L), streptavidin Alexa Fluor 488 conjugate, streptavidin Alexa Fluor 594 conjugate, Hoechst, DAPI and rhodamine-phalloidin were from Molecular Probes (Eugene, OR).

Solid phase binding assays

Immulon® 2 HB (ThermoLabsystems, Franklin, MA) microtiter strips with breakaway wells were coated directly with 50 μl of the indicated concentrations of versican, versican G1 domain, or TSP1 by incubating overnight at 4°C in Dulbecco's PBS without Ca^{2+}, Mg^{2+}. Non-specific sites were blocked with 3% (w/v) BSA in Dulbecco's PBS (DPBS) at room temperature for 1 hour. [^{125}I]-TSP1 (0.5 μg ml^{-1}, 50 μl per well) was added alone or in the presence of increasing concentrations of the indicated unlabelled ligands as competitor in Dulbecco's PBS, containing 0.5% (w/v) BSA, 0.1 mM phenylmethylsulfonyl fluoride with or without Ca^{2+}, Mg^{2+} and incubated at 37°C for 3 hours. The wells were washed with the same cold buffer and the bound radioactivity was quantified using a gamma counter (Packard BioScience Company, Downers Grove, IL). Self-displacement binding experiments were analyzed using Scafit version 2.4 of the LIGAND programs (Munson and Rodbard, 1980). For detection of binding to immobilized TSP1, wells were incubated with biotinylated versican or aggrecan link domains and, following washing, with [^{125}I]-streptavidin at 1 μg ml^{-1}.

Cell culture

Human aortic smooth muscle cells (HASMC, Cambrex Bio Science Walkersville, MD) were maintained in SmGM-2 (Clonetics, Wakersville, MD) medium supplemented with 5% FCS, gentamicin/amphotericin B, insulin (5 μg ml^{-1}), hFGF-2 (2 ng ml^{-1}) and hEGF (1 ng ml^{-1}). HASMC were plated at a density of 1×10^6 cells per 75-cm^2 flask, passaged weekly by trypsinization with a solution of 0.05% trypsin, 0.02% EDTA and they were split at a 1:4 ratio. Cells were used for experiments up to passage 7.

Immunofluorescent staining and confocal microscopy

Cells were grown in 8-well glass chamber slides (45×10^4 cells per well, per 300 μl medium) 2-3 days before use and treated as described in the figure legends. Subsequently, the incubation medium was removed and the cells were fixed with 4% paraformaldehyde solution in PBS for 7 minutes at room temperature and then blocked with HBSS containing 4% BSA for 30 minutes. Samples were stained with a solution containing biotinylated hyaluronan-binding protein (5 μg ml^{-1}) and/or an appropriate primary antibody (2-10 μg ml^{-1}) in HBSS containing 4% BSA for 1 hour at room temperature. The chamber slides were washed three times with HBSS and then incubated with solution containing secondary antibodies at a dilution as recommended by the manufacturer for 1 hour at room temperature. Hoechst 33258 was added to the final incubation solution to stain nuclei. The slides were washed four times with HBSS and then rinsed in water. The cells were imaged using an

Olympus IX70 fluorescence microscope and a Spot Insight cooled digital camera (Diagnostic Instruments, Sterling Heights, MI). Images of each field were captured at three wavelengths and with phase contrast illumination. Monochrome images were combined using Corel Photopaint. Quantitative image analysis was done using Image Pro Plus software (Media Cybernetics). For analysis using confocal microscopy, cells were plated onto 12 mm glass coverslips in a 24-well tissue culture dish, grown for 2-3 days and treated as described in the figure legends. Cells were rinsed with PBS and fixed for 12 minutes with 4% paraformaldehyde in PBS and then rinsed three times with PBS. To permeabilized, cells were treated with 1% Triton X-100 in 0.02% BSA/PBS for 2 minutes and rinsed three times with PBS. Cells were incubated in blocking buffer (20% goat serum in 2% BSA/PBS) for 30 minutes and then stained with primary and appropriate secondary antibody as mentioned above. Rhodamine-phalloidin used to stain F-actin was diluted according to the manufacturer's procedure and placed on the cells for 30 minutes. Finally, cells were washed three times with PBS, incubated with DAPI (1:10,000 in PBS) for 5 minutes, washed three times with PBS and mounted onto microscope slides using Gel/Mount (Biomeda Corp., Foster City, CA). Stained cells were visualized on a Zeiss (Thornwood, NY) Axiovert 100M microscope equipped with a 100×/1.3 oil objective and confocal microscopy was performed using a Zeiss LSM510 scanning laser microscope. Three-dimensional maximal projection images were generated from Z-stacks. Multi-tracking configurations were used to eliminate cross-talk of fluorochromes.

TSP1 immunoassay

HASMC were plated in a 96-well plate at 5×10^3 cells per well and allowed to grow for 48 hours in SmGM-2 medium containing 5% FBS and treated appropriately for the described experiments with SmGM-2 medium containing 1% FBS alone or with 5 μg ml^{-1} poly I:C for indicated time points. Concentration of TSP1 in conditioned medium was determined by ELISA. Briefly, 96-well plates (Nunc) were coated with heparin-BSA (5 ng per well), blocked with Tris-BSA buffer (50 mM Tris, 1% BSA, 0.02 mM PMSF, 150 mM NaCl, 1 mM $CaCl_2$, pH 7.8) for 30 minutes at room temperature, followed by incubation with 50 μl samples or standard (10-1000 ng ml^{-1} TSP1) for 2 hours at 37°C. The wells were then aspirated and washed and 50 μl of anti-TSP1 rabbit polyclonal antibody (R187) diluted 1:500 in the Tris-BSA buffer was added to each well and incubated for 2 hours at 37°C. After washing three times the wells were then incubated with goat anti-rabbit IgG (Kirkegaard and Perry) diluted with the Tris-BSA buffer to 1:1000 and incubated for 1 hour at room temperature. The plate was then washed three times and bound antibody conjugate was detected with o-phenylenediamine dihydrochloride (Sigma).

Reverse transcription and real-time qPCR

Total RNA from HASMCs treated or untreated with 5 μg ml^{-1} poly I:C for 2 or 24 hours was isolated using the Trizol method (Invitrogen) and was quantified with a Beckman DU640 spectrophotometer. After DNase digestion, 5 μg of total RNA was reverse transcribed using Superscript II (Invitrogen) and random primers. PCR products were synthesized from cDNA using TaqMan probe sets for elastin, versican, TSP1 and HPRT (Applied Biosystems). Steady state mRNA levels were determined using a DNA Engine Opticon I Continuous Fluorescence Detection System (M.J. Research, Waltham, MA). Samples were incubated 95°C for 2 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Taqman PCR amplification was performed in a 50 μl reaction volume using 280 ng of cDNA and probe and primer concentrations of 250 nM and 900 nM respectively. Relative gene expression levels were quantified using the comparative threshold (C_t) method with HPRT1 serving as the endogenous reference gene.

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