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The evolving role of thrombospondin-1 in hemostasis and vascular biology

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Abstract. Thrombospondin-1 (TSP1) is a multi-domain, multi-functional glycoprotein synthesized by many cells. Matricellular TSP1 modulates cell adhesion and proliferation. TSP1 is involved in angiogenesis, inflammation, wound healing and cancer. As a major platelet protein, for a long time it was postulated to control hemostasis *via* platelet aggregate stabilization. However, these *in vitro* findings have been questioned in the absence of corroborating clinical data and of obvious hemostatic defects in TSP1 gene-deficient mice. Yet, the past few years have provided indices to implicate TSP1 in hemostasis. In clinical studies, a correlation exists between a well-defined TSP1 polymorphism and a significant risk of myocardial infarction. At the same time, recent *in vivo* animal model data imply TSP1 in the multimer size control of von Willebrand factor, in smooth muscle cell regulation and in vascular perfusion. These findings shed new light on the role of TSP1 in hemostasis and prothrombotic vascular pathologies. (Part of a Multi-author Review)

Keywords. Matricellular proteins, cell adhesion, platelet, wound healing, ADAMTS-13, CD47, CD36.

Introduction

Although thrombospondin-1 (TSP1) is primarily referred to as a protein involved in angiogenesis and wound healing, its abundance in platelet α-granules has stimulated multiple investigators to study the role of TSP1 in platelet function and hemostasis. This review will mainly focus on recent advances made in this field, but will also deal with new insights in the function of TSP1 in the vessel wall, in relation to its role in (patho)physiological vascular changes, relevant for the occurrence of thrombosis.

TSP1 is part of a thrombospondin family, consisting of five members. The five thrombospondins can be subdivided into two subgroups, depending on their molecular organization. TSP1 and TSP2 are part of the first subgroup and are trimers; TSP3, TSP4 and TSP5 are pentamers. TSP1 resides on chromosome 15, TSP2 on chromosome 6 and TSP4 on chromosome 5. TSP1, TSP2 and TSP4 have been associated with myocardial infarction [1]. All thrombospondins display a high degree of homology, but they are differentially expressed in various tissues, in agreement with their distinct promoters, suggesting at least partially different functions for each member of the thrombospondin family.

TSP1 was first described in 1971 as a high molecular weight glycoprotein secreted from blood platelets upon thrombin activation [2]. TSP1 is synthesized by megakaryocytes as a major constituent of platelet α-granules, accounting for 3% of the total platelet protein mass (30–100 μg/10^9 platelets, i.e. about 10^5 molecules per platelet) and for 25% of the proteins secreted by platelets, upon thrombin activation. Upon degranulation, about half of the released TSP1 sticks

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to the cell surface. In physiological conditions, TSP1 is present in plasma at concentrations ranging from 60 to 300 ng/ml [3]. In the vessel wall, it is constitutively synthesized by endothelial cells, fibroblasts and smooth muscle cells [4, 5].

TSP1 is a multifunctional, matricellular glycoprotein, containing interacting domains for a large variety of adhesive proteins, cell receptors and enzymes, and it is involved in numerous biological processes, including cell adhesion, migration and proliferation, cell-cell interactions, angiogenesis, tumor cell metastasis, inflammation, atherosclerosis, hemostasis and thrombosis [6–8]. The multi-functionality of TSP1 has complicated correct understanding of TSP1 function, since, depending on its concentration and cellular localization in different tissues, it interacts with different receptors to a variable degree, triggering seemingly opposite biological effects. Recent observations in several animal models, however, are progressively unraveling the biology of the multipotent thrombospondin family.

Structure and functional domains of TSP1

TSP1 is a homotrimeric glycosylated protein of M_r 450 kDa (Fig. 1). Mature polypeptide chains contain 1152 amino acids (aa) (180 kDa), linked by disulfide bonds between cysteine 252 and 256 [9]. Each TSP1 subunit comprises N- and C-terminal globular domains (N and G domains) and a thin connecting strand [10, 11]. The N domain (approximatively 200 aa) is cleaved from TSP1 by several proteinases, including thrombin, plasmin, cathepsin G, elastase, trypsin and chymotrypsin. This domain, also designated as heparin binding domain, contains several groups of basic amino acids and is characterized by its affinity for heparin, residing in three amino acid sequences within the N domain [12]. The cleaved N domain exists both in a soluble state [13] and can associate with activated platelet membranes [14]. This domain functionally diverges from and even antagonizes the functions of native TSP1 [15]. Its structure has recently been solved and is discussed in detail by Carlson et al. in an accompanying paper [16]. The N domain interacts with fibrinogen (Fg) [17, 18], glycosaminoglycans (related to heparin), calreticulin, low-density lipoprotein receptor-related protein and integrins $\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_6\beta_1$ and $\alpha_9\beta_1$ (Fig. 1) [19–22].

The connecting strand is composed of a procollagen (PC) homology domain, three properdin-like domains (or Type 1 repeats; TSR1 s) and three EGF-homology domains (or Type 2 repeats; TSR2 s) (Fig. 1 and [16]). The CSVTCG sequence within the two last TSR1 s was reported to be involved in CD36 binding [23], but the activity of the synthetic CSVTCG peptide may not mimic the native sequence in the three-dimensional structure of the TSR, as both cysteines in CSVTCG are part of separate disulfide bonds and the threonine is O-glycosylated [24]. An adjacent sequence, GVXXRXX, seems to contain the CD36-binding motif [25]. TSR1 was found to interact with heparin and glycosaminoglycans [26], but also with fibrin(o-gen) [27].

Transforming growth factor-β (TGFβ) modulates cell growth and proliferation. TSP1 activates latent TGFβ and protects it against inactivation through α_2-macroglobulin and decorin proteoglycan [28, 29]. Two sequences in TSR1 interact with TGFβ: RFK in the activation of latent TGFβ and GGWSHW, binding to active TGFβ [30]. TSR2 s contain 55 residues, including 6 cysteines, and present 24% homology with epidermal growth factor (EGF), but also with coagulation factors IX and X and with tissue plasminogen activator. Several ligands interact with the connecting strand, including plasminogen [31], fibronectin, laminin [32] and type I to type IV collagens [33]. Type V collagen was found to associate with the procollagen domain and the first TSR1 domain of TSP1 [34].

The C-terminal portion of TSP1 comprises 7 type III repeats (TSR3) and a G domain [16]. TSR3 s confer to TSP1 the capacity to bind up to 33 Ca^{2+} cations (11 per chain) [35]. The binding of Ca^{2+} to TSP1 profoundly influences its structure by stabilizing the C-terminal portion. In the absence of Ca^{2+}, the length of the stalk increases with TSR3 module linearization [10, 11], and reactive sites for thrombin and trypsin are exposed. Each TSP1 monomer contains 3 free thiols among the 12 cysteines of the TSR3 modules. Depending on the Ca^{2+} concentration, these residues will differentially pair and modify the C-terminal portion folding. The C-terminal proximal TSR3 module contains an RGDA sequence, potentially allowing interactions of TSP1 with platelet integrins $\alpha_{IIb}\beta_3$, $\alpha_v\beta_3$ and $\alpha_5\beta_1$. The accessibility of the RGDA sequence and, therefore, the adhesive properties of TSP1 are thus tightly related to the Ca^{2+}-ion concentration and disulfide bond matching [36, 37]. Sun et al. [38] have shown that controlled reduction of disulfide bonds in TSP1 by dithiothreitol increases its RGDA-dependent cell-adhesive properties.

Free thiols and a Ca^{2+}-modulated C-terminal region confer to TSP1 the capacity to form covalent bonds with other proteins, such as thrombin. TSP1 multimerization, due to disulfide bond exchange, has also been described [36]. More recently, protein disulfide isomerase (PDI) secreted by activated platelets was found to catalyze disulfide exchange reactions on the platelet surface [39]. Thus, the action of PDI revealed the cryptic RGDA on TSP1 [40], catalyzing

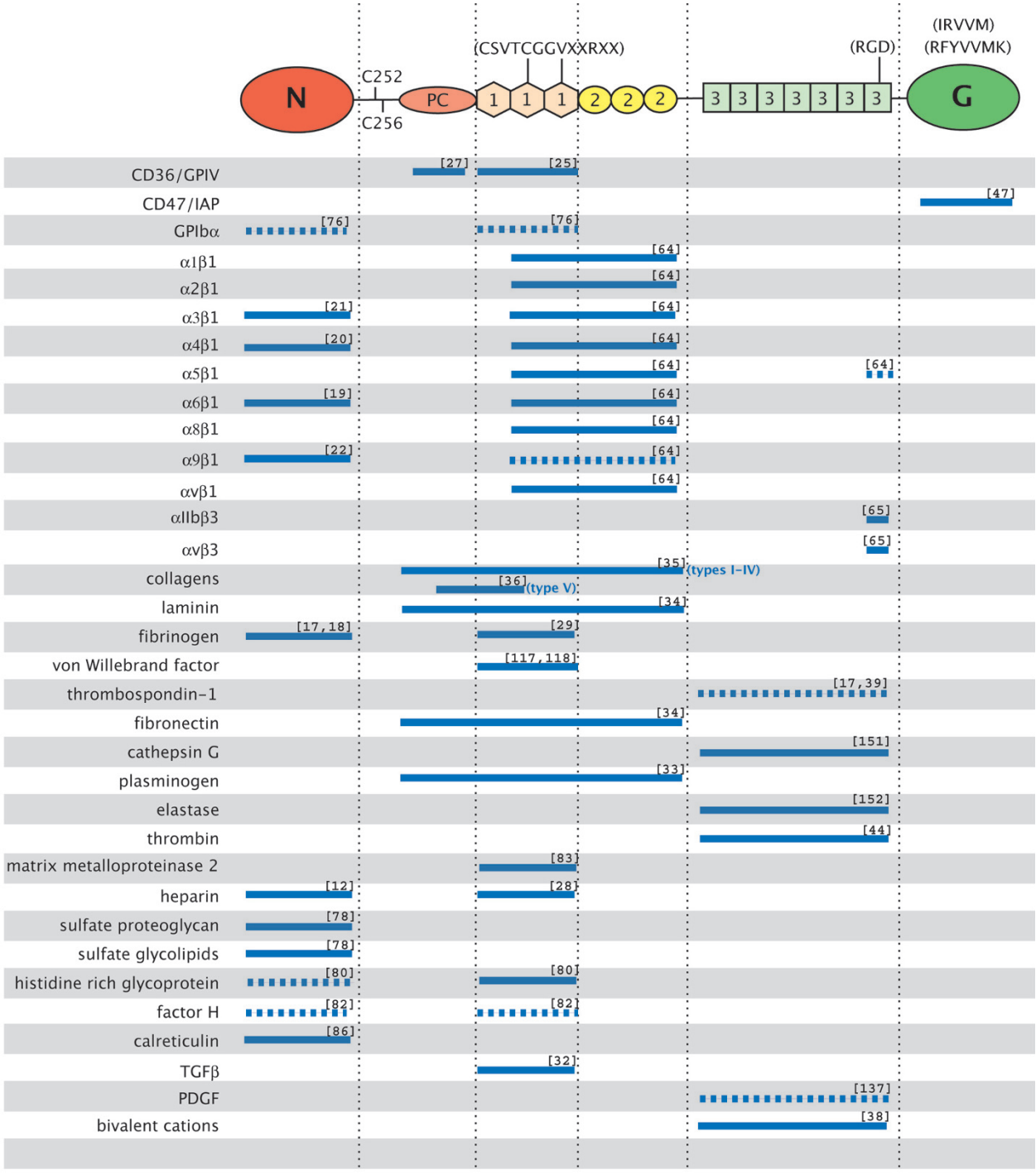

Figure 1. Functional domains of TSP1. Single chain of TSP1 schematically shown as a series of functional domains: globular N-terminal domain (N), procollagen (PC), thrombospondin repeats TSR1 (hexagons), TSR2 (ovals), TSR3 (squares) and the globular C-terminal domain (G). Both cysteines (C252, C256) involved in interchain disulfide bonding are indicated. Amino acid sequences involved in binding of CD36 (CSVTCGGVXXRXX), CD47 (RFYVVMK, IRVVM) and integrins (RGD) are noted over their respective location in the molecule. Underneath are listed the numerous molecules with known (solid lines) or suspected (dashed lines) interaction site(s) on TSP1.

formation of a TSP1-thrombin/antithrombin complex [41].

The G domain is specific to the TSP family (TSP1-5) [42] and is involved in platelet agglutination [43] and aggregation [43]. The G domain also contains sequences, able to induce adhesion of endothelial cells and several cancer cells. Gao et al. [44] found that the integrin-associated-protein (IAP/CD47), a receptor for TSP on many cell types, including platelets, specifically interacts with RFYVVM on TSP1.

Presence of TSP1 on platelets

The platelet membrane expression of TSP1 has been studied *via* several approaches, including electron microscopy [45], I^{125} labeling of the activated platelet surface [46] and immunofluorescence [47]. These approaches all detected weak TSP1 binding on the surface of resting platelets. Strong agonists, such as thrombin or the calcium ionophore A23187, in contrast, trigger abundant presence of TSP1 on the platelet surface, mostly colocalized with Fg and $\alpha_{IIb}\beta_3$ integrin, within macromolecular structures [45, 48]. Hourdillé et al. [48] showed that surface-bound TSP1 was drastically decreased by EDTA. Quantitative studies, using monoclonal antibodies and purified I^{125}-TSP1, showed about 1000–2000 TSP1 molecules on resting platelets, independent of the presence of bivalent cations [49], but 14000–16000 TSP1 molecules on the activated platelet surface for 70–80% dependent on bivalent cations [49, 50]. Thus, at least two types of receptors seem to be involved in TSP1 binding on the surface of platelets: non-inducible and inducible TSP1 receptors. More recently, Saumet et al. [51] demonstrated that thrombin activation leads to rapid and specific association of a large amount of secreted TSP1 with the actin cytoskeleton. This association was inhibited by cytochalasin D and by the MAII antibody directed against the N domain of TSP1, but was independent of CD36 or $\alpha_{IIb}\beta_3$.

Platelet receptors for TSP1

CD36

Glycoprotein IV or CD36 is an 88-kDa adhesive receptor present on platelets, endothelial cells, monocytes and several tumor cells. It is present at about 20000 copies on the platelet plasma membrane and at 20000 copies in α-granules. CD36 contains carbon hydrates to 26% of its mass and 10 N-glycosylation sites [52]. CD36 interacts with oxidized low-density lipoproteins (and corresponds to the scavenger receptor on macrophages), but also with TSP1 [53] and collagen I and V [54, 55]. Asch et al. [56] showed that CD36 binding to TSP1 and collagens depends on phosphorylation/dephosphorylation of Thr92 by protein kinase C. Constitutively phosphorylated on resting platelets, CD36 can bind collagen, but not TSP1, whereas dephosphorylation during platelet activation abrogates collagen binding and induces TSP1 binding. The binding of TSP1 to CD36 leads to intracellular signals [57]. CD36 seems to participate in TSP1 expression on the surface of activated platelets. Electron microscopy revealed membrane co-localization of the two proteins [58]. Moreover, several studies reported that monoclonal antibodies against CD36 could inhibit TSP1 expression on the platelet membrane, and co-immunoprecipitation experiments suggested the existence of a direct interaction, independent of divalent cations [59]. However, only 40–50% inhibition of surface binding of TSP1 could be achieved with anti-CD36 antibodies, and CD36-deficient platelets have a normal surface expression of TSP1, indicating that CD36 is not the unique platelet receptor for TSP1 [60].

Integrins

TSP1 directly interacts with β_1 integrins, including $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$ and $\alpha_9\beta_1$. This interaction is mediated by the second and third TSR1 and TSR2 s, and the N domain [19–22, 61]. TSP1 also interacts with β_3 integrins. It possesses a cryptic RGDA motive allowing potential binding with $\alpha_v\beta_3$ and $\alpha_{IIb}\beta_3$. Characterization of the TSP1 interaction with platelet integrins led to contradictory results, in particular regarding $\alpha_{IIb}\beta_3$ binding. Lawler and Hynes [62] showed that $\alpha_v\beta_3$ and $\alpha_{IIb}\beta_3$ complexes isolated from platelet membranes were retained by TSP1 immobilized on Sepharose columns and that GRGDSP specifically inhibited binding. Using purified ligands, Kowalska and Tuszynski [63] confirmed a weak interaction ($K_d = 3.77$ μM), highlighting requirement of the bivalent cations Mg^{2+} and Ca^{2+}. The authors also described similar interactions between TSP1 and $\alpha_2\beta_1$. Other groups, on the contrary, reported no interaction between TSP1 and $\alpha_{IIb}\beta_3$ [64, 65]. Monoclonal anti-$\alpha_{IIb}\beta_3$ antibody 10E5 inhibited the binding of TSP1 to the surface of activated platelets by 94%, whereas another anti-$\alpha_{IIb}\beta_3$ antibody, AP-2, had no effect [49]. The abrogation of TSP1 binding observed by Wolff et al. [66] could be due to inhibition by 10E5 of the binding of Fg and fibronectin, both also TSP1 ligands, to $\alpha_{IIb}\beta_3$. Such an interpretation is supported by the demonstration that AP-2 inhibited the binding of exogenous Fg to the integrin by 85%, but could not prevent membrane expression of endogenous Fg upon platelet activation with thrombin.

CD47/IAP

CD47 is a member of the immunoglobulin (Ig) receptor superfamily, present on the surface of many mammalian cells. It has an M_r of 50 kDa and possesses an extracellular IgG domain, five hydrophobic transmembrane domains and a short cytoplasmic tail [67]. CD47 was reported to modulate the function of several integrins, including $\alpha_v\beta_3$ [68], $\alpha_{IIb}\beta_3$ [69] and $\alpha_2\beta_1$ [70]. Frazier et al. [71] showed that CD47 couples with heterotrimeric G proteins. The binding of the RFYVVMWK peptide to CD47 was shown to modulate the integrin-dependent platelet spreading on immobilized Fg. Such processes contribute to platelet aggregation through $\alpha_{IIb}\beta_3$ activation and synergisti-

cally modulate $\alpha_2\beta_1$-dependent platelet spreading and aggregation to collagen [72].

GPIb

Jurk et al. [73] showed that immobilized TSP1 supports firm platelet adhesion in LMWH- or hirudin-anticoagulated blood at high shear rates (1600–1900 s^{-1}). Adhesion was strongly inhibited by antibodies against GPIbα or by glycocalicin (the soluble N-terminus of GPIbα), but was independent of VWF, $\alpha_2\beta_1$, $\alpha_{IIb}\beta_3$, $\alpha_v\beta_3$, CD47 and only slightly decreased by anti-CD36 antibodies. The authors suggested that, at high shear rates, encountered in stenosed vessels, TSP1 could act as a backup for GPIbα, i.e. be an alternative substrate to VWF. They also noticed that platelet adhesion to TSP1 was reversed by the chelation of Ca^{2+}. Interestingly, Onitsuka et al. [74], in an earlier study, described a weak and transient interaction of flowing platelets with immobilized TSP1, purified in the presence of 5 mM EDTA. Since platelet adhesion to TSP1 is highly sensitive to Ca^{2+} concentration, this suggests possible microenvironmental regulation of the TSP1-GPIbα interaction.

Glycolipid sulfate

Roberts et al. [75] described a strong and specific affinity of TSP1 for the galactosyl-ceramide-I3-sulfate-type glycolipid sulfate ($K_d = 10$ nM) present in the platelet surface membrane. This interaction is partially resistant to EDTA and strongly inhibited by heparin. Human small cell lung carcinoma cell lines attach to TSP1 and manifest extended neurite outgrowth, a process dependent on the cooperation between the $\alpha_v\beta_3$ integrin and sulfated glycolipids [76], but no such role in platelet function has specifically been reported for platelet-TSP1 interactions.

Other ligands of TSP1 with possible function in hemostasis

TSP1 interacts with other molecules with known or putative regulatory functions in atherothrombosis, including adiponectin [77], histidine-rich glycoprotein [78], galectin [79], complement factor H [80], matrix metalloproteinase-2 [81], lysosomal integral membrane protein II (LIMPII), a protein member of the CD36 family [82], the heavy chain of elastase-activated coagulation factor V [83] and calreticulin-low density lipoprotein receptor-related protein receptor complex [84].

TSP1 interactions with platelet ligands

Fibrinogen

As soon as Fg was identified as the receptor for the lectin activity of activated platelets, and TSP1 was found to be responsible for this lectin activity [85], several studies reported stable and specific interactions between the two proteins [17, 27, 64, 86, 87], with a high affinity (K_d's of 3, 4 to 156 nM) and independent of Fg binding to $\alpha_{IIb}\beta_3$. TSP1 and Fg co-localize on the surface of activated platelets [45, 47]. Several studies confirmed that Fg contributes to TSP1 expression on activated platelets: TSP1 binds Fg on fixed platelets [88]; the binding of the monoclonal anti-TSP1 antibody 5G11 on the surface of thrombin-activated platelets was inhibited by 50% by polyclonal anti-Fg Fab [49]; the agglutination of washed platelets by TSP1 was inhibited by an excess of Fg [89]. In counterpart, activated platelets from afibrinogenaemic patients have normal TSP1 amounts on their surface, indicating redundancy in the players responsible for TSP1 expression on platelets.

Fibrin

TSP1 interacts (non)-covalently with fibrin, through transglutamine bridges formed by coagulation factor XIIIa [90, 91]. This binding involves a Lys- and Glu-rich region of the connecting stalk [92] and TSR1 motifs [27] of TSP1, and sequences α113–126 and β243–252 of Fg, respectively. Electon microscopy showed that fibrin fibers are more numerous and thinner in the presence of TSP1 [90]. Bale and Mosher [93] suggested that TSP1 accelerates fiber growth by serving as a trifunctional branching unit during network formation. Hemostatic consequences of this remain uncertain. The resistance of the connecting stalk to proteolytic degradation suggests that this region of TSP1 could serve as a cell adhesion site (via CD36 binding to TSR1 modules) on the fibrin clot even during fibrinolysis by plasmin.

Homotypically to TSP1

TSP1 can self-associate into covalent polymers with a molecular mass of several million Daltons. TSP1 polymerization is independent of bivalent cations [94]; polymers do not pre-exist in α-granules, but are found in the supernatant of activated platelets and during TSP1 purification [36, 94]. TSP1 could be a substrate for PDI, potentially triggering TSP1 multimerization. Lahav et al. [95] showed that soluble TSP1 can interact with immobilized TSP1, and another study showed that TSP1 immobilized on polystyrene beads triggered agglutination of beads, in a viscosimeter, sensitive to EDTA during shearing [17]. Only scarce data point to a functional role for TSP1 multimerization. Boot et al. [94] demonstrated that it was responsible for the lectin activity of TSP1 that induces erythrocyte agglutination. Tuszynski et al. [96] suggested that the dimerization of TSP1 (M_r 902 kDa) was responsible for CD36-dependent platelet aggre-

gation. It seems than the agglutinating activity of TSP1 multimers is directly related to the multivalent character of these structures.

TSP1 and platelet function

Role in platelet adhesion

TSP1, synthesized by endothelial cells, fibroblasts and smooth muscle cells, is a constituent of the vascular wall, where it interacts with other vascular factors, including fibronectin, proteoglycans, collagens, laminin and von Willebrand factor (VWF). In static conditions, the adhesion of resting platelets onto glass or fibronectin-coated coverslips is inhibited by TSP1 [97]. In contrast, Tuszynski and Kowalska [98] observed platelet adhesion and spreading onto TSP1-coated plastic coverslips. Platelet adhesion was increased by divalent cations (Mn^{2+}, Mg^{2+} and Ca^{2+}) and was dependent on $\alpha_{IIb}\beta_3$ and/or $\alpha_2\beta_1$. Agbanyo et al. [99] demonstrated that in the absence of Ca^{2+}, TSP1 is non- or anti-adhesive, whereas it supports platelet adhesion in the presence of Ca^{2+}. Platelet adhesion onto TSP1 in flow was maximal at a shear rate of 1600 s^{-1} and adhesion depended on $\alpha_{IIb}\beta_3$ and $\alpha_2\beta_1$, but not on CD36 or $\alpha_v\beta_3$.

TSP1 in platelet aggregation: conflicting *in vitro* and *in vivo* data

The contribution of TSP1 to platelet aggregation was first evidenced *in vitro*, using monoclonal antibodies directed against particular domains of the molecule [100–102], or peptides and recombinant fragments of TSP1 [103–105], through models with isolated platelets. In addition, membranes or activated fixed platelets bearing Fg were used [88, 96, 106]. These approaches concluded that TSP1 stabilizes the irreversible phase of platelet aggregation concomitantly with platelet secretion. The N domain of TSP1 seemed to contribute strongly: monoclonal antibody MAII directed against N domain inhibited platelet aggregation and secretion, induced by thrombin [18, 103]. However, other regions of the TSP1 TSR3 [18], the G domain [102] and TSR1 domains containing CSVTCG [105] also seemed to be involved in platelet aggregation. The peptide RFYVVMWK also induced platelet aggregation through the FcRγ-chain signaling pathway, coupled to agglutination. The latter pathway was independent of signaling events and of GPIbα or $\alpha_{IIb}\beta_3$. Surprisingly, no pathway was mediated *via* IAP [107]. Others reported that RFYVVMWK induced platelet aggregation through an $\alpha_{IIb}\beta_3$ and Fg-independent mechanism [108]. Several groups have shown that TSP1 is more effective in aggregation at low concentration of thrombin (<0.05 U/ml) [18, 100, 103]. Levy-Toledano et al. [109] showed that platelets from patients with the inherited gray platelet syndrome with absent α-granule components (including TSP1) aggregate normally at high concentrations of thrombin (>0.1 U/ml), but are impaired at lower doses. Nonetheless, TSP1 null platelets in plasma responded normally to various concentrations of thrombin, ADP, TxA_2 and equine tandon collagen, when stirred in the cuvette of an aggregometer, in physiological concentrations of plasma protein ligands [110, 111].

Mostly these studies were performed under nearly static conditions and not under the physiological flow environment and shear stress encountered in the normal circulation. Yet, shear stress-controlled studies have shown that TSP1 is involved, indirectly or directly, in the formation of interplatelet bridges. Interactions between TSP1 and Fg are instrumental in this process, and determine the size and reversibility of platelet aggregates. A model from Asch and Nachman [112] suggested that TSP1 bound to a primary receptor (IAP or CD36) and to Fg, stabilizing the binding of the latter to $\alpha_{IIb}\beta_3$, thereby strengthening platelet aggregation. Secreted TSP1 was found to reinforce interplatelet interactions in flowing blood and accelerate aggregation, through Fg-TSP1-Fg and direct TSP1-TSP1 cross-bridges [17]. In these studies, soluble TSP1 induced aggregation of Fg-coated beads dose-dependently, at physiological shear rates (100–2000 s^{-1}), through the N domain. The interaction of soluble TSP1 with Fg-$\alpha_{IIb}\beta_3$-coated beads resulted in the formation of aggregates via Fg-TSP1-Fg cross-bridges. Such was evidenced *via* cross-bridge formation between $\alpha_{IIb}\beta_3$-Fg on one particle and free $\alpha_{IIb}\beta_3$ on a second particle, interactions blocked by the RGD mimetic Ro 44–9883. Yet, TSP1 binding to Fg already bound to $\alpha_{IIb}\beta_3$ blocked the ability of this occupied Fg to recognize another $\alpha_{IIb}\beta_3$ molecule, although cross-bridging occurred because the bound TSP1 interacted with another Fg molecule on a second particle. As represented in Figure 2, these studies provided a model for the reinforcement by secreted TSP1 of interplatelet interactions in flowing blood.

Flow-controlled studies of the interaction between platelets and endothelium have further shown that TSP1 secreted from activated platelets behaves as an autocrine and paracrine activator through its capacity to bind platelet receptors IAP and CD36, and induces cellular intracellular signaling [113], also important for platelet interactions with endothelial cells. Such is relevant, since resting platelet adhesion to inflammatory vascular endothelium is thought to play a causal role in secondary thrombus formation. These authors showed, using human platelets and platelets from wild-type and $CD47^{-/-}$ mice, that at low shear rate,

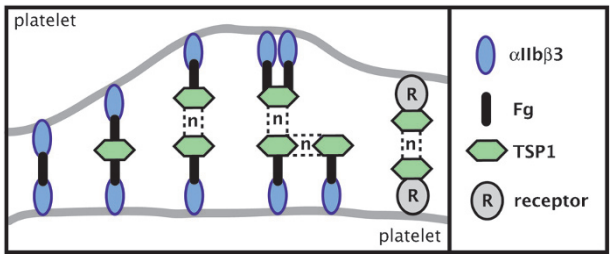

Figure 2. Model for TSP1 in platelet-platelet interactions. Stabilization of platelet-platelet bonds in flow depends on TSP1 binding to $\alpha_{IIb}\beta_3$-bound Fg present on separate platelets, as well as on a direct cross-bridging role for TSP1, linking receptors on adjacent platelets.

platelet CD47 significantly contributed to platelet adhesion on TNFα-stimulated endothelial cells. CD47 bound the G domain of endothelial TSP1, inducing activation of platelet $\alpha_{IIb}\beta_3$ integrin, in turn linking the endothelial receptors ICAM-1 and $\alpha_v\beta_3$. Platelet CD36 and GPIbα were also involved because platelet incubation with blocking mAbs directed against each of these 2 receptors significantly decreased platelet arrest. Anti-CD47 treatment of platelets did not further decrease the adhesion of anti-CD36-treated platelets, underscoring that the CD36/TSP1 interaction could trigger the CD47-dependent pathway.

TSP1 and COATed platelets

COATed platelets are a subpopulation of collagen- and thrombin-activated platelets with a highly procoagulant surface enriched in surface-bound serotonin-derivatized factor V, Fg, fibronectin, VWF, TSP1 and α_2-antiplasmin under the control of transglutaminases and factor XIIIa [114]. TSP1 and Fg are the receptors for serotonin on platelets forming stable and multivalent complexes on the cell membrane.

TSP1 and VWF

TSP1 interactions with VWF

Barabino et al. [115] have shown that purified VWF specifically interacts with immobilized TSP1. By surface plasmon resonance spectroscopy, Onitsuka et al. [74] measured a dissociation constant $K_d = 3.97 \times 10^{-7}$ M for the interaction between immobilized TSP1 and VWF. TSP1 bound to the A3 domain of VWF *via* its TSR1 domains [116, 117]. In our own experience, FITC-TSP1 can interact with VWF immobilized on latex beads in the presence of 2 mM Ca^{2+}. Yet, we failed to detect agglutination of VWF beads by soluble TSP1, perhaps due to the incapacity of TSP1 to cross-bridge VWF on adjacent beads, or due to the reductase activity of TSP1 on VWF multimers.

The physiological relevance of VWF-TSP1 interactions is unclear. Agbanyo et al. [99] have shown that TSP1 inhibits the adhesive functions of VWF in citrated blood. We have performed perfusions of calcein-labeled platelets in reconstituted blood (washed red blood cells in tyrode buffer) over surfaces of TSP1 and VWF. Platelet adhesion to coated human TSP1 was slightly shear rate-dependent; the addition of soluble VWF to physiological concentrations did not stimulate platelet deposition on the TSP1 surface, but reduced platelet binding to TSP1 at all shear rates investigated (937, 1250 and 1875 s^{-1}) (Fig. 3). Similar perfusions over coated VWF surfaces revealed strong platelet recruitment, not affected by TSP1 addition to the perfusate, at 3 μg/ml (Fig. 3). These findings provide evidence for the existence of direct interactions between TSP1 and VWF, but exclude that these interactions stimulate platelet recruitment. Although these studies fit with the report by Jurk et al. [73] of strong binding of platelets to TSP1 via GPIbα, they also reveal what appears to be a slight competition between TSP1 and VWF for binding to their common platelet receptor GPIbα. Pimanda et al. [116] reported an increase in collagen- and VWF-mediated aggregation of the TSP1 null platelets under both static and shear conditions in a PFA-100 device. These findings suggest that TSP1 competition with VWF for GPIbα binding could result in a mild downregulation of GPIbα function in dynamic flow conditions.

Control of VWF multimer size by TSP1

During the past few years, the group of Chesterman and Hogg published several papers identifying TSP1 as a reductase, capable of modulating the multimerization state and therefore the hemostatic activity of VWF [117]. The authors demonstrated that the incubation of purified TSP1 with VWF resulted in formation of thiol-dependent complexes of TSP1 and VWF, generation of new thiols in VWF, and reduction of the average VWF multimer size. The VWF-reducing activity of TSP1 was shown to center on a free thiol at Cys974 located in the Ca^{2+}-binding G domain [118]. The administration of TSP1 to wild-type Balb/c mice resulted in a reduction of the average multimer size of plasma VWF [117]. The average multimer size of plasma VWF determined in TSP1 null mice backcrossed in C57Bl/6 background was significantly smaller than in wild-type C57Bl/6 mice [116]. Results, however, seem to vary with the mouse background in which these studies were performed. Hence, the size distribution pattern of the plasma and platelet VWF multimers were found identical in TSP1 null mice back-crossed in the Swiss background compared to Swiss wild-type mice [110], pointing to genetic variation and/or genetic modifiers for this property.

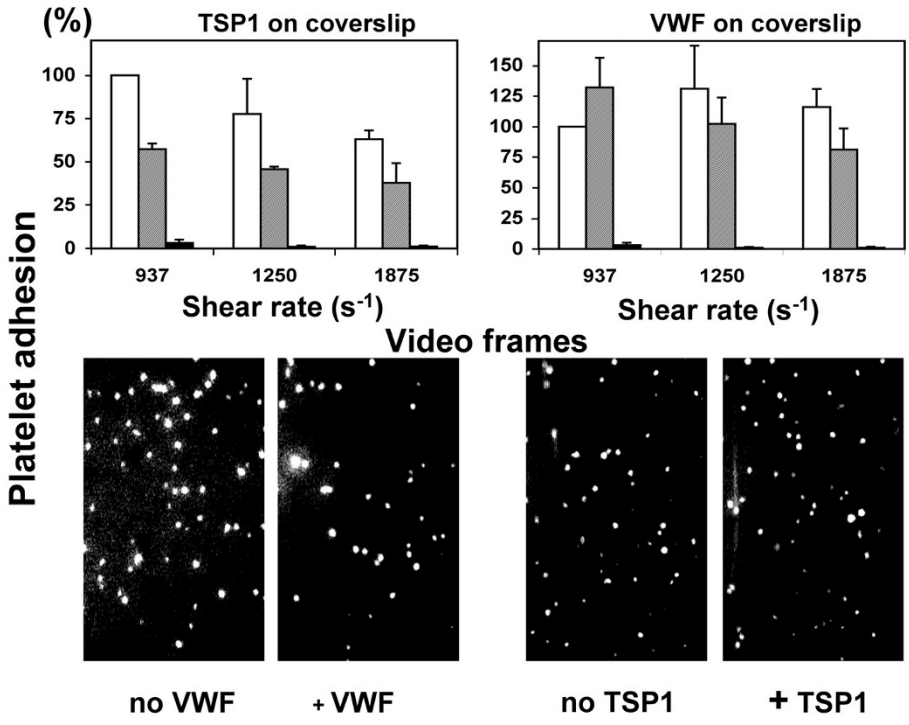

Figure 3. TSP1-VWF interactions during platelet perfusion studies. Human platelet adhesion to glass coverslips coated with purified human TSP1 (50 μg/ml), or human VWF (25 μg/ml), as indicated, at increasing shear rates, expressed proportional to the number of adhering platelets after perfusion for 5 min at the lowest shear rate (937 s^{-1}), set as 100%. Perfusion with calcein-labeled platelets was performed in reconstituted blood at 10000 platelets/μl and in the absence (open bars) or presence (grey bars) of VWF in the perfusate (10 μg/ml, left upper panel) or TSP1 (3 μg/ml, right upper panel). Black bars represent the percentage adhesion on non-coated coverslips.

TSP1 protects VWF from degradation by ADAMTS-13

Stable thrombi can form in arterioles of mice lacking both VWF and Fg, indicating that other vascular or platelet ligands, such as fibronectin, can contribute to platelet adhesion and aggregation [119, 120]. Diffuse alveolar hemorrhage was observed in TSP1 null mice in normal hemostatic conditions in the original 129Sv homogeneous background. Since, however, no abnormal thrombotic phenotype was reported in TSP1 null mice in baseline hemostatic conditions [111] and in a back-crossed Swiss background [110], at first, it seemed that TSP1 has no obvious function in hemostasis, other than controlling the VWF multimer size in plasma of mice on selected backgrounds, such as described above [116].

In vitro evidence for competition between ADAMTS-13 and VWF

Plasma contains a VWF-cleaving protease which assures VWF processing of ultralarge VWF multimers implicated in thrombotic thrombocytopenic purpura in patients with congenital or acquired deficiency for this enzyme [121]. This enzyme, ADAMTS-13, a member of the metalloproteinase family, contains several TSRs. It cleaves ultralarge VWF when multimers are temporarily retained on the surface of the endothelium where VWF is synthesized [122]. The activity of ADAMTS-13 on endothelial surfaces has been reproduced in flow chambers during perfusion with (reconstituted) blood [110]. This has shown that platelet binding to ultralarge VWF multimers enhances tensile forces on the adhered VWF, facilitating its cleavage by ADAMTS-13. TSP1 dose-dependently inhibited the ADAMTS-13-mediated VWF processing at concentrations readily achieved upon platelet secretion [110]. These findings were compatible with competition between ADAMTS-13 and TSP1 for binding to stretched VWF multimers and with a protective role for TSP1 in the ADAMTS-13-mediated processing of VWF multimers.

In vivo models of vascular inflammation and thrombosis

Less platelet recruitment was noted on inflamed endothelium of $Tsp1^{-/-}$ mice in conditions where such platelet recruitment depends on the participation of VWF [110]. This defect could be restored by injection of human TSP1 but also by administration of neutralizing anti-ADAMTS-13 antibodies. Hence, absence of TSP1 stimulated ADAMTS-13 to intensely degrade vascular VWF, involved in recruitment of platelets, a phenotype corrected by TSP1 or anti-ADAMTS-13 antibodies, both inactivating the protease, *via* enzyme competition and complexation, respectively.

Upon vascular injury of $Tsp1^{-/-}$ mouse arterioles, normal platelet aggregation but defective thrombus adherence was observed: large thrombi easily detached in the direction of the blood flow. This phenotype

was also restored by injection of human TSP1 or by administration of neutralizing anti-ADAMTS-13 antibodies [110]. In addition, raising plasma TSP1 to saturating concentrations, inactivating ADAMTS-13, even led to a prothrombotic tendency with vessel occlusion times, shorter than in controls. These findings illustrated that ADAMTS-13 also trims subendothelial and/or thrombus-associated VWF, controlling thrombus stability. Hence, TSP1 does not affect shear stress-induced thrombus formation but thrombus stability by competing with ADAMTS-13 (Fig. 4). Full inactivation of ADAMTS-13 by high TSP1 concentrations triggers a phenotype in agreement with the thrombotic tendency observed in patients with congenital or acquired deficiency of this enzyme [121].

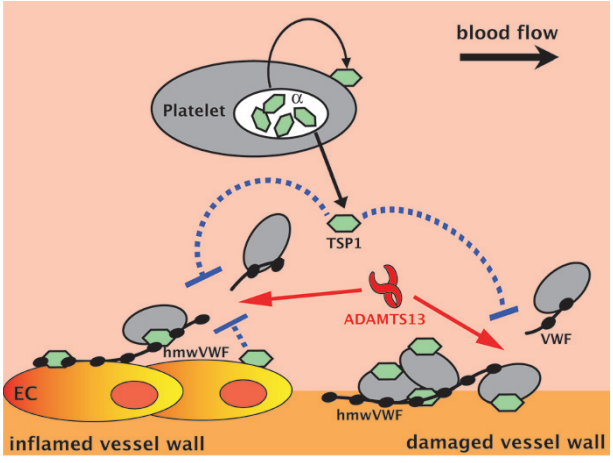

Figure 4. The role of TSP1 in the shear stress-controlled proteolysis of VWF by ADAMTS-13. Endothelial cell activation or injury exposes VWF multimers, capable of platelet binding to VWF A1 domains (A domains are represented on VWF multimer chains, hmwVWF, as separate knots). VWF, stretched in a shear field, is exposed to plasma ADAMTS-13, hydrolyzing VWF A2 domains, thus shortening the length of released VWF. TSP1, secreted from activated platelets, binds to the platelet surface, but also competes with ADAMTS-13 for binding to VWF A domains, slowing down VWF hydrolysis and thus protecting the high-affinity binding between hmwVWF and single platelets and platelet-rich thrombi.

Vascular TSP1 and vessel wall disease

TSP1 single-nucleotide polymorphisms and vascular disease

A single-nucleotide polymorphism (SNP) in TSP1 has been associated with cardiovascular disease. The TSP1 SNP is located in the coding region for the carboxy-terminal between the TSR2 s and TSR3 s, encoding an N700S substitution. The mutation is recessive, rare in whites and is associated with increased risk for myocardial infarction [1]. The N700S substitution alters the calcium-binding properties of TSP1, since a Ser in position 700 may function as a coordinating residue in a Ca^{2+}-binding site [123]. ^{700}S-TSP1 is increased in platelets from carriers and enhances platelet aggregation *in vitro* [124]. This finding may constitute the mechanism underlying myocardial infarction in carriers, due to increased thrombus formation. However, vascular expression of ^{700}S-TSP1 in smooth muscle cells (SMCs), endothelial cells (ECs) or inflammatory cells may further modulate normal vessel wall function, as suggested by several observations, linking TSP1 with vascular disease. Thus, TSP1 is strongly upregulated in rat carotid arteries after balloon angioplasty [125], at sites of hyperplasia, in hypercholesterolemic lesions and in the adventitia of diabetic rats, suggesting a role for TSP1 in the increased vulnerability of diabetics to restenosis [126–128]. SMC proliferation induced by high glucose depends on TSP1 upregulation [129], and vasculopathy has been associated with persistent elevation of TSP1 in human cardiac allografts [130]. Accordingly, neutralization of TSP1 by antibodies accelerated re-endothelialization and reduced neointima development after carotid artery denudation [131]. In counterpart, the study in $Tsp1^{-/-}$ mice of healing myocardial infarcts has revealed a role for TSP1 in protecting the non-infarcted myocardium from fibrotic remodeling [132]. In models of ischemic injury, $Tsp1^{-/-}$ mice manifest increased nitric oxide (NO)-mediated tissue perfusion, implicating TSP1 as a physiological antagonist of NO-mediated vasodilatation and tissue perfusion. Elegant work has shown that CD47 is the TSP1 receptor, limiting NO-mediated vascular smooth muscle relaxation [133]. TSP1 is a potent naturally occurring angiogenesis inhibitor. The lower density of vasa vasorum in diabetic aortas with increased levels of TSP1 [126] may, therefore, further promote ischemia in the inner layers of large vessels, adding to thrombogenicity induction.

TSP1 and vascular cells

Atherosclerosis and restenosis are strongly linked to endothelial damage, causing recruitment of circulating platelets and leukocytes, as well as release of cytokines and growth factors. Already after early endothelial cell activation, SMCs migrate from the media towards the lumen where they proliferate. In endothelial cell cultures, TSP1 upregulates expression of VCAM1, ICAM1 and P-selectin, promoting monocyte attachment [134] *via* the G domain of TSP1, which interacts with CD47. Cell culture studies demonstrated that SMC proliferation and migration were also stimulated by TSP1. Thus, Majack et al. showed that platelet-derived growth factor (PDGF) induced expression in SMCs of high amounts of TSP1. Cell-surface TSP1 further potentiates SMC responses

to PDGF [135], epidermal growth factor or angiotensin-2. TSP1 binds to platelet-derived growth factor (PDGF), directing it to its receptor on SMCs [136]. Recent studies support a PDGF-independent role for TSP1 in SMC proliferation, implicating $\alpha_3\beta_1$ [137], $\alpha_v\beta_3$ [138] and CD47 [139] in signaling pathways involving ERK1/2 and S6 kinase. The interaction of TSP1 with platelet integrins further mediates platelet-induced SMC proliferation [140]. TSP1 also affects SMC migration in Boyden chamber and transwell assays, identifying it as a chemoattractant for SMCs, a property probably located in the TSP1 G domain [141], interacting with $\alpha_v\beta_3$, $\alpha_2\beta_1$ and CD47. Intracellular signaling relies on activation of focal adhesion kinase, phosphatidyl inositol-3-kinase, p21 Ras, ERK1/2 and p38 [137]. TSP1-induced SMC migration on gelatin also relies on MMP-2 activation [142]. In a model of neointima formation, after carotid artery ligation without endothelium damage, TSP1 facilitates SMC proliferation and migration, and the development of a neointima. In the absence of TSP1, proliferation and migration of SMCs are reduced, and a more fibrotic collagen-rich neointima develops [143]. Therefore, by participating in the regulation of SMC proliferation and migration *via* different TSP1 receptors and metabolic pathways, TSP1 is instrumental in vascular (re)modeling (Fig. 5) and its associated thrombogenicity during vascular lesion recovery.

TSP1 also potently antagonizes NO-stimulated SMC responses [144]. This activity may be mediated by TSR1 s and a CD36-dependent cGMP signaling pathway, also responsible for TSP1-induced inhibitory effects on endothelial cells [144]. However, CD47 seems to be the only TSP1 receptor absolutely necessary for this inhibition [133]. In models of ischemic injury, TSP1 limits the NO-mediated vasodilatation and tissue perfusion, and hence is a factor implicated in ischemia, itself in turn also linked to thrombogenicity.

TSP1 in inflammation, wound healing and vascular disease

Wound healing in the vessel wall involves disturbed hemostasis, macrophage-mediated inflammation, angiogenesis, and cell proliferation and migration. TSP1 is strongly and quickly upregulated in excisional wounds, initially as a result of platelet degranulation in the wound, later as a result of synthesis by macrophages and fibroblasts [145]. TSP1 antisense oligonucleotides could reduce the number of TSP1-positive macrophages in spite of normal total tissue TSP1 levels and macrophage numbers [146]. Decreased TSP1 expression did not affect macrophage infiltration, although wound healing was delayed in TSP1

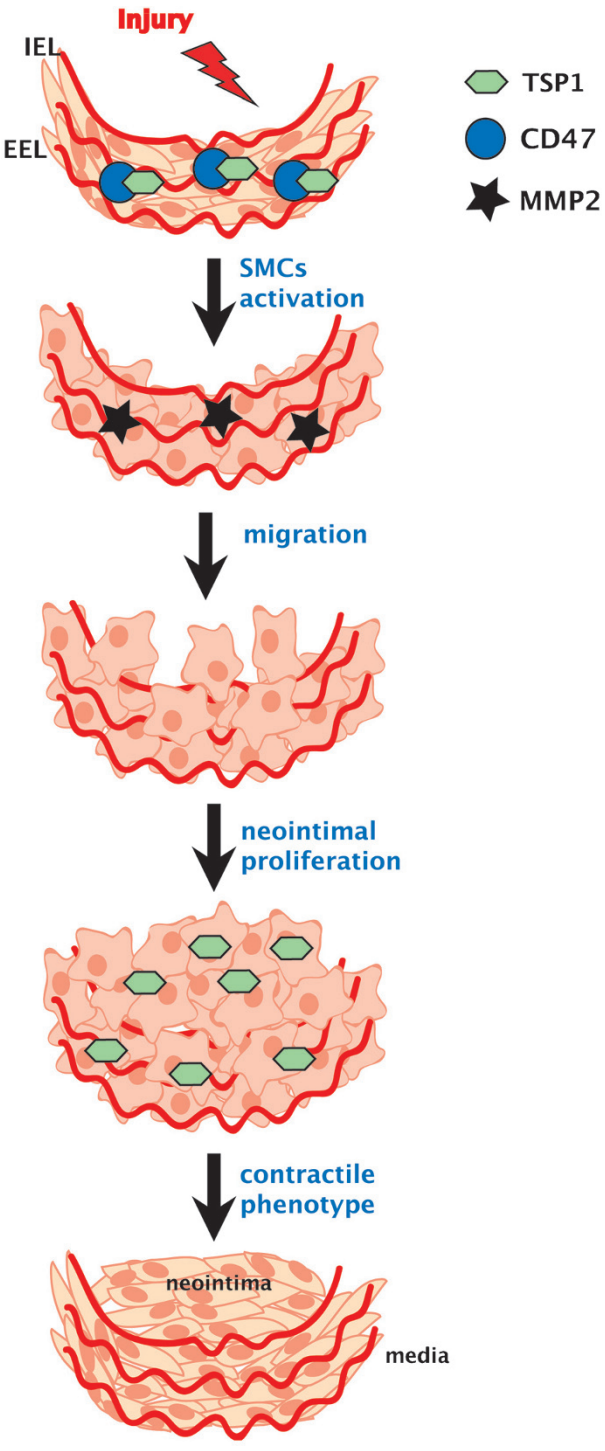

Figure 5. Schematic representation of the role of TSP1 in smooth muscle cell function. After vascular injury, the expression of TSP1 is quickly upregulated. By interacting with SMCs, most likely *via* CD47, TSP1 activates SMCs, leading to media thickening, MMP-2 activation, SMC mobilization and formation of a neointima, strongly positive for TSP1. Eventually SMCs return to the contractile phenotype, also in neointima, probably under control of the high matricellular levels of TSP1.

antisense-treated animals. $Tsp1^{-/-}$ mice show enhanced leukocyte infiltration, pneumonia with macrophages and neutrophils [111], and mild inflammation in the pancreas. This pro-inflammatory phenotype is attributed by some authors to a deficient conversion of latent to active TGFβ1 [147]. The physiological relevance of TGFβ1 activation by TSP1 is, however, a subject of controversy, since TGFβ1 can also be activated by other means. Moreover, albeit contradictory, TGFβ1-independent effects of TSP1 on inflammatory cells have been described.

TSP1 is secreted by and adheres to neutrophils, monocytes, macrophages and T-cells [148], and several TSP1 receptors are present on inflammatory cells. CD36, CD47 and $\alpha_v\beta_3$ downregulate pro-inflammatory cytokine release by dendritic cells (DCs), through CD47 selectively downregulating IL12 production by monocytes, and inhibiting DC maturation independent of TGFβ [149]. TSP1-CD47 interactions seem to contribute to refractoriness of mature DCs, avoiding further activation, i.e. suggesting an important role for TSP1 in the resolution of the inflammatory response. Consistent with a moderating role for TSP1 in inflammation, TSP1 mediates phagocytosis of apoptotic cells [150] *via* a ligand bridge $\alpha_v\beta_3$/CD36/TSP1 between macrophages and apoptotic cells. Furthermore, phagocytosis of intact cells hinders release by these cells of pro-inflammatory cytokines involved in tissue repair. The contribution to atherosclerotic lesion development of TSP1 has not yet formally been reported. The chronic inflammatory state of this vascular disease can be anticipated to involve vascular cellular cross-talk in atherosclerotic lesions, determining the size and composition of the developing plaque. Together with its role in SMC migration and proliferation, reported above, TSP1 therefore seems to be a determinant in the progression of atherogenesis, a major risk factor in the development of myocardial infarction, triggered by plaque rupture.

Conclusions

Released as a soluble protein from platelets or present as a matricellular protein in the vessel wall, TSP1 therefore directly and indirectly participates in cellular cross-talk, relevant for hemostasis and vascular integrity. Upon acute injury and during chronic inflammatory vascular injury, TSP1 is strongly upregulated as a result of secretion and transcription factor regulation. This suggests that thrombospondins are more critical in disease than in health. The further study of the role of TSP1 and other thrombospondins and their functional mutants in atherothrombosis, restenosis, vascular remodeling, chronic vascular inflammation, blood vessel regeneration in ischemia and wound healing, especially in pathological conditions (metabolic syndrome, diabetes, myocardial infarction, atherosclerosis, platelet secretion defects) therefore seems warranted. The availability of $Tsp1^{-/-}$ mice enables these studies in a relevant animal model, *via* application of several techniques such as reciprocal bone marrow transplantations, hydrodynamic gene transfer of relevant thrombospondin mutants, tissue- and cell-specific overexpression of thrombospondins and of such mutants in acute and chronic disease models. These studies will ultimately document the therapeutic applicability of TSP1 neutralization in the management of chronic vascular pathologies.

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