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Thrombospondin-1: a physiological regulator of nitric oxide signaling

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Abstract. Thrombospondin-1 is a secreted protein that modulates vascular cell behavior via several cell surface receptors. *In vitro*, nanomolar concentrations of thrombospondin-1 are required to alter endothelial and vascular smooth muscle cell adhesion, proliferation, motility, and survival. Yet, much lower levels of thrombospondin-1 are clearly functional *in vivo*. This discrepancy was explained with the discovery that the potency of thrombospondin-1 increases more than 100-fold in the presence of physiological levels of nitric oxide (NO). Thrombospondin-1 binding to CD47 inhibits NO signaling by preventing cGMP synthesis and activation of its target cGMP-dependent protein kinase. This potent antagonism of NO signaling allows thrombospondin-1 to acutely constrict blood vessels, accelerate platelet aggregation, and if sustained, inhibit angiogenic responses. Acute antagonism of NO signaling by thrombospondin-1 is important for hemostasis but becomes detrimental for tissue survival of ischemic injuries. New therapeutic approaches targeting thrombospondin-1 or CD47 can improve recovery from ischemic injuries and overcome a deficit in NO-responsiveness in aging. (Part of a Multi-author Review)

Keywords. Ischemic injury, tissue perfusion, angiogenesis, hemostasis, blood flow, nitric oxide, vascular smooth muscle, platelets.

Introduction

Thrombospondin-1 (TSP1) is a large glycoprotein that is synthesized and secreted by many cell types in response to injury and certain growth factors [1]. TSP1 is expressed transiently at various sites during development, but high expression in the healthy adult is largely restricted to megakaryocytes, circulating platelets, and sites of tissue remodeling or injury. Elevated tissue expression of TSP1 is also characteristic of several chronic diseases, including diabetes and atherosclerosis [2–5]. Relevant to the following discussion, it is important to recognize that normal plasma contains low but significant levels of circulating TSP1 (100–200 pM) [6]. Thus, vascular endothelium is constantly exposed to low levels of TSP1. Furthermore, our recent work shows that functionally significant levels of TSP1 are present in certain normal tissues even if they may not be detectable by standard immunohistology [7]. This review focuses on our recent advances in understanding the role of both physiological and pathological levels of TSP1 as a negative regulator of nitric oxide (NO) signaling in vascular cells and the implications of this function in cardiovascular physiology and pathology.

In order to respond to physiological or pathological levels of TSP1, cells must express appropriate TSP1 receptors. Unfortunately, elucidation of the cardiovascular functions of TSP1 has been complicated by the large number of TSP1 receptors and extracellular matrix (ECM) ligands of TSP1 in these tissues. TSP1

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interacts with at least nine receptors on endothelial cells, including α3β1, α4β1, α6β1, α9β1, and αvβ3 integrins [8–13], the LDL receptor-related protein (LRP) in association with calreticulin [14], heparan sulfate proteoglycans [15], CD36 [16], and CD47 [17]. These interactions give rise to diverse endothelial cell responses. The integrins α3β1 and αvβ3, CD36, and CD47 similarly mediate distinct responses to TSP1 in vascular smooth muscle cells (VSMCs) [18–20]. In platelets, CD36 and CD47 appear to be the major direct receptors for TSP1, although TSP1 binding to fibrinogen may also allow indirect binding to the major platelet integrin αIIbβ3 [21, 22].

The daunting task of reconciling all of these receptor interactions with specific cellular responses to TSP1 has been somewhat simplified by recognizing that some receptors require different TSP1 concentration thresholds to elicit responses and by evidence that the expression and/or activity of some TSP1 receptors is highly regulated at the cellular level or by conformational regulation of TSP1 through its interactions with other ECM components [23].

The major TSP1 receptors relevant to this review are CD36 and CD47. CD47 is a ubiquitously expressed transmembrane protein that is present on all cardiovascular cell types and platelets (reviewed in [24]). CD36 is a scavenger receptor and fatty acid translocase that is more abundantly expressed on capillary and post-capillary venule endothelium [25], but is also expressed at lower but functional levels in large vessel endothelial cells [26]. CD36 is also highly expressed on VSMCs (vascular smooth muscle cells) and platelets [25, 27].

TSP1 as an angiogenesis inhibitor

Historically, endothelial cells were the first vascular cell type shown to be responsive to TSP1 *in vitro*, and this led to the perception that the primary function of TSP1 is as an angiogenesis inhibitor [28–31]. Endothelial cell adhesion, growth, motility, and survival are clearly regulated by TSP1, and TSP1 was the first identified endogenous inhibitor of angiogenesis (reviewed in [32, 33]). TSP1 can be demonstrated in the basement membrane of quiescent blood vessels in the skin but is absent in areas of endothelial cell outgrowth and angiogenesis [34]. It is known to potently inhibit endothelial cell pro-angiogenic responses, including adhesion, proliferation, and migration *in vitro*, and angiogenesis *in vivo* [28, 29, 35].

TSP1 at nanomolar concentrations blocks endothelial cell growth and migration stimulated by pro-angiogenic factors such as fibroblast growth factor-2 (FGF2) and vascular endothelial growth-factor (VEGF) [28, 29]. TSP1 also induces apoptosis of endothelial cells when pro-survival factors are limiting [36]. Consistent with these *in vitro* results, TSP1 potently inhibits FGF2-stimulated angiogenesis in standard *in vivo* assays, including the chick chorioallantoic membrane assay and the rat corneal pocket assay [29, 37]. In contrast, a pro-angiogenic activity of TSP1 was reported in the rabbit cornea [38]. Subsequent studies have mapped both pro- and anti-angiogenic domains in TSP1 and confirmed pro-angiogenic responses of the N-domain of TSP1, suggesting that differential proteolytic processing of the protein may yield both stimulatory and inhibitory TSP1 fragments (Fig. 1).

The anti-angiogenic activities of TSP1 are generally regarded to contribute to its effects on tumor growth. Mice lacking TSP1 are not inherently more susceptible to spontaneous tumor formation but show increased incidences of some tumor types and decreased survival when crossed into a p53 null background [39]. A syngeneic melanoma placed in TSP1 null mice grew more rapidly and had more metastatic potential than similar tumors in wild-type mice. Conversely, mice engineered to overexpress TSP1 in skin or mammary epithelia show decreased cancer incidence in these tissues [40, 41]. In humans, malignant progression of some tumor types is associated with loss of TSP1 expression [42–46]. Conversely, human tumor cells engineered to constitutively express TSP1 grew much slower and had lower microvascular densities as xenografts in mice than the parental tumors that did not make TSP1 [47, 48]. However, the relatively high concentrations of TSP1 required to inhibit endothelial cell responses *in vitro* until recently has remained difficult to reconcile with its potent activity *in vivo* and the published evidence that low circulating levels of TSP1 in tumor-bearing mice can inhibit angiogenesis and growth of nascent metastatic lesions in the same animal [49].

The reported anti-angiogenic activities of TSP1 in various tumor models led to current efforts to develop cancer therapeutics based on TSP1. Studies employing recombinant TSP1 domains and synthetic peptides demonstrated that inhibition of FGF2-stimulated angiogenesis is mediated by the type 1 repeats of TSP1 and that this process in part requires the cell surface receptor CD36 [16, 37]. A peptide mimetic drug based on a CD36-binding sequence in the type 1 repeats of TSP1 (ABT-510) has been found to increase survival of tumor-bearing mice and dogs and has progressed to human phase II clinical trials [50].

In addition to endothelial cells, TSP1 may modulate angiogenesis through its effects of VSMCs. Angiogenesis once initiated will not persist without recruitment of VSMCs. VSMCs associated with capillaries,

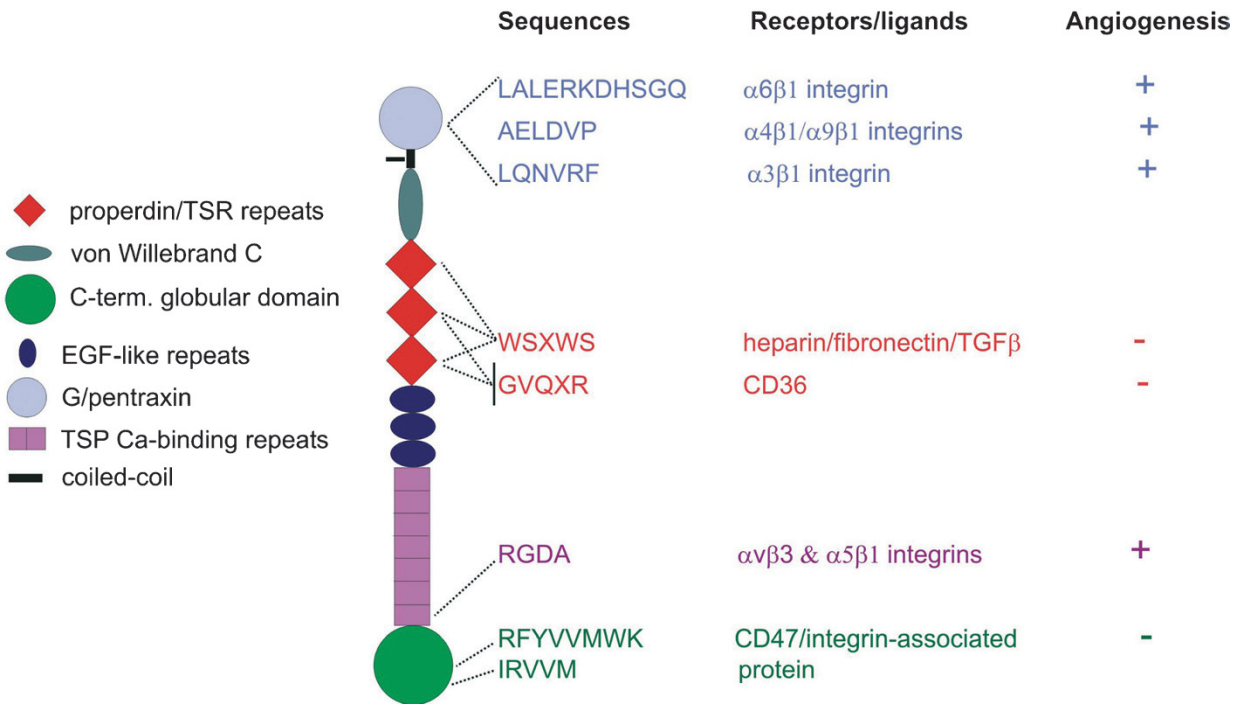

Figure 1. Pro- and anti-angiogenic functions of TSP1 and its endothelial cell receptors. The N domain of TSP1 stimulates angiogenic responses via α3β1 [9], α4β1 [10], α6β1 [12], and α9β1 [13]. The type 1 repeats contain two distinct anti-angiogenic sequences that interact with sulfated glycoconjugates [136], fibronectin [137], TGFβ [138], and the receptor CD36 [16]. The type 3 repeats contain an RGD sequence that interacts with α5β1 and αvβ3 integrins [8, 139] This sequence can stimulate endothelial cell adhesion, but its role in angiogenesis is unclear. The C-terminal G domain interacts with CD47. Some CD47-binding peptides derived from this domain inhibit angiogenesis [140], and CD47 is necessary for anti-angiogenic activities of TSP1 in the context of NO signaling [86].

termed pericytes, directly interface with endothelial cells but do not form a complete investing layer as found in larger arterioles and arteries [51]. In contrast to endothelial cells, TSP1 was initially found to stimulate motility and proliferation of VSMCs and, based on TSP1 antibody effects, was proposed to be necessary for VSMC proliferation [52–54]. Further studies demonstrated that, like endothelial cells, TSP1 has biphasic effects on VSMC proliferation and motility [55]. Based on a comparison of murine wild-type and TSP1 null VSMCs, we found that TSP1 is not necessary for proliferation but is permissive for platelet derived growth factor (PDGF)-stimulated cell migration [19]. Because recruitment of VSMCs and pericytes to nascent vasculature is necessary for stable angiogenic responses [56–58], TSP1 may modulate angiogenesis by acting on both vascular cell types.

Monocytes and macrophages are increasingly recognized to play important roles in angiogenesis [59, 60]. Studies of angiogenesis in incisional wounds in TSP1 null mice also imply a role of macrophages as a target for TSP1 [61]. TSP1 null mice did not show the predicted increase in tissue blood vessel densities but instead showed a deficit in macrophage recruitment. Other studies have revealed diverse functions of TSP1 in both innate and adaptive immunity [62]; so myeloid cells should also be considered as potential mediators of TSP1 effects on angiogenesis.

Nitric oxide

Nitric oxide (NO) is a bioactive gas produced through the conversion of L-arginine to L-citrulline by three nitric oxide synthases (nNOS/NOS1, iNOS/NOS2, and eNOS/NOS3) [63, 64]. eNOS is the predominant NOS isoform expressed in endothelial cells and platelets. Vascular NO can also be increased under hypoxic conditions by nitrite reductase activities of several proteins, including hemoglobin, myoglobin, and xanthine oxidase [65–67]. The biological significance of NO was first recognized when it was identified as the active component of endothelial-derived relaxing factor. This factor was defined by the observation that removing the endothelium from arterial segments ablated their relaxation in response to acetylcholine. Ignarro and colleagues demonstrated that bubbling pure NO gas into a medium bath containing aortic segments recapitulated the degree of relaxation induced by acetylcholine when applied to endothelium-competent segments [68–71]. Removal

of the endothelium did not impede the relaxation by NO treatment, implying that NO exerts it effects directly upon VSMCs.

NO was subsequently found to stimulate soluble guanylate cyclase (sGC) in VSMCs, which elevates cytoplasmic cGMP levels. cGMP activates cGMP-dependent protein kinases (cGKs) and gated ion channels to regulate the ability of VSMCs to contract (Fig. 2). Relaxation of VSMCs by NO increases blood vessel diameter and blood flow. As the functional moiety of nitroglycerine and related nitrovasodilators, NO has been utilized therapeutically for treatment of myocardial ischemia secondary to coronary artery atherosclerosis [72, 73].

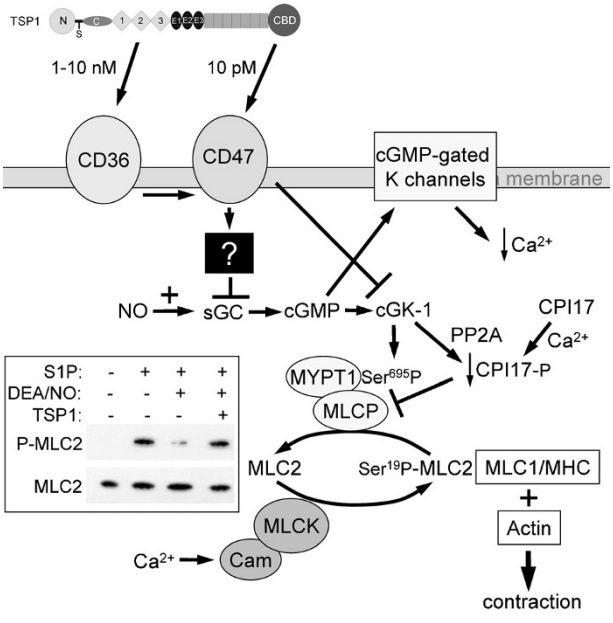

Figure 2. Regulation of vascular smooth muscle cell contraction by TSP1. Agonist-stimulated VSMC contraction involves Ca-dependent activation of myosin light chain kinase (MLCK) and inactivation of myosin light chain phosphatase via its regulatory subunit MYPT1 and phosphorylation of its inhibitor CPI-17. MLCK phosphorylates the regulatory subunit of myosin (MLC2) on Ser^{19} to activate myosin/actin-mediated VSMC contraction. NO relaxes VSMC via increasing cGMP, which simultaneously activates cGMP-gated potassium channels and cGMP-dependent protein kinase-I (cGK-1). The former reduces intracellular calcium levels, and the latter phosphorylates MYPT1 on Ser^{695} and leads to dephosphorylation and inactivation of CPI-17 via protein phosphatase 2A (PP2A) [141]. TSP1 signaling through CD36 and CD47 prevents cGMP accumulation by inhibiting sGC and simultaneously inhibits cGK-1 to prevent downstream signaling [22, 86]. Thus, TSP1 increases MLC2 phosphorylation (inset) and increases VSMC contractility by blocking the relaxing activities of NO.

A number of additional physiologic functions have been ascribed to NO under both resting and stress conditions. Low concentrations of NO physiologically promote vascular cell survival and proliferation [74]. However, these effects of NO are biphasic, with higher amounts causing cell growth arrest and, at NO levels produced by iNOS in activated inflammatory cells, cell death [75]. Angiogenic responses in both endothelial and VSMCs such as proliferation, adhesion, and migration are increased by NO from <1 to ~ 50 nM [26, 55, 76] In contrast, these same responses are inhibited by >100 nM NO. The stimulatory signaling of low-dose NO can be blocked using inhibitors of sGC, whereas the inhibitory activities of higher NO levels are mediated by activation of p53 and dephosphorylation of MAP (mitogen-activated protein) kinases via MKP1 [77].

In a 3D *ex vivo* model of wound healing, muscle tissue explants in a type I collagen matrix demonstrate vascular cell invasion and migration through the collagen matrix. These effects are dramatically stimulated by exogenous NO or L-arginine. In contrast, the vascular outgrowth of tissue explants into a collagen matrix is inhibited by the nonspecific NOS inhibitor N-nitro-L-arginine methyl ester (L-NAME) [26]. Consistent with these results, *in vivo* models of wound healing have shown a role for NO and L-arginine in promoting wound healing [78–80].

Inhibition of NO signaling by TSP-1

We recently discovered that muscle tissue explants from TSP1 null mice demonstrate enhanced vascular outgrowth in a collagen matrix relative to wild-type tissue explants [81]. This differential response was enhanced in the presence of L-arginine or an exogenous slow-releasing NO donor such as DETA/NO [26]. Conversely, outgrowth was blunted by the NOS inhibitor L-NAME, and again the degree of inhibition of vascular outgrowth was less in TSP1 null explants as compared to wild-type. NO-stimulated increases in endothelial cell proliferation, adhesion, and migration were potently blocked by exogenous TSP1 at picomolar concentrations. Additionally, primary murine endothelial cells from TSP1 null animals demonstrated significantly greater increases in proliferation and adhesion when compared to wild type cells. Likewise, NO-stimulated accumulation of cGMP was profoundly sensitive to exogenous TSP1, with picomolar concentrations being sufficient to completely block NO stimulation of sGC. Recombinant TSP1 type 1 repeats, which interact with CD36, mimicked TSP1 to block NO/cGMP signaling in endothelial cells and to inhibit an NO-stimulated accumulation of cGMP. In contrast, a recombinant fragment from the N-terminal domain of TSP1 did not block NO-driven effects in endothelial cells and had no effect on NO-stimulated cGMP levels. Significantly, in the presence of NO

endothelial cells become more than 100-fold more sensitive to the inhibitory activity of TSP1. The physiologic importance of these findings was underscored by the finding that TSP1 null endothelial cells consistently demonstrate increased basal levels of cGMP compared to wild-type cells. Thus, endogenous TSP1 functions as a tonic antagonist of endogenous NO signaling in endothelial cells.

It was not clear that antagonism of NO signaling by TSP1 would extend to VSMCs, since some earlier work had indicated reciprocal responses of VSMCs and endothelial cells to TSP1 (e.g. inhibition of endothelial cell proliferation and chemotaxis but stimulation of the same in VSMCs). Yet we found that picomolar concentrations of TSP1 demonstrated similar inhibition of NO-stimulated VSMC proliferation, adhesion, and migration as were observed with endothelial cells [55]. Thus, NO reprograms VSMC responses to parallel those of endothelial cells, and this may facilitate the coordinated outgrowth of endothelial cells and pericytes required for a persistent angiogenic response.

The above responses to NO are important for long-term regulation of VSMCs during vascular remodeling, but a more important acute physiological role of NO in these cells is to regulate vascular smooth muscle tone to control blood flow [82–85]. On a molecular level, VSMC contracture involves interactions between myosin and the actin cytoskeleton. Agonist-mediated contraction of VSMCs is mediated by calcium/calmodulin-dependent activation of myosin light chain kinase, which phosphorylates the myosin light chain-2 (MLC2) regulatory subunit and leads to increased assembly of stress fibers containing F-actin and increased contractility via myosin-actin interactions (Fig. 2). NO relaxes VSMCs by increasing cGMP, which acts through cGMP-gated potassium channels to lower intracellular calcium and through cGK to activate myosin light chain phosphatase (MLCP), which dephosphorylates the regulatory MLC2 subunit and thereby prevents myosin/actin contraction. We found that TSP1 disrupts this activity of NO and prevents dephosphorylation of MLC2 in the presence of NO (Fig. 2) [7]. To assess contracture *in vitro*, VSMCs were placed into a 3D collagen matrix and stimulated to contract using serum or the specific agonist sphingosine 1-phosphate. NO predictably relaxed cells that had been stimulated to contract by these agonists. Exogenous TSP1 at 2 nM completely blocked the ability of NO to relax contracting VSMCs. Thus, in addition to its long-term effects on VSMC growth and motility, TSP1 acutely regulates smooth muscle tone by antagonizing the vasorelaxing activity of NO.

Inhibition of NO/cGMP signaling via CD47

Pursuing the molecular mechanism by which TSP1 blocks NO-driven vascular cell responses, we confirmed that recombinant type 1 repeats of TSP1 and peptides derived from the same region of TSP1 that interact with the cell surface receptor CD36 could effectively inhibit NO signaling in endothelial and VSMCs [26, 55]. Recombinant fragments from the pro-angiogenic N-terminal region of TSP1 did not block NO signaling in vascular cells. However, an exclusive role for CD36 as the relevant TSP1 receptor was inconsistent with our observation that muscle explants from CD36 null mice show similar inhibition by TSP1 of NO-stimulated vascular outgrowth as wild-type explants [86]. A role for the TSP1 receptor CD47 was shown by the inability of TSP1 to inhibit vascular outgrowth from CD47 null muscle explants. Consistent with this data, peptides based on a sequence in the C-terminal domain of TSP1 that were previously shown to be CD47 ligands [17] and recombinant proteins containing the C-terminal domain potently inhibited NO-driven signaling in vascular cells comparable to whole TSP1 [86]. CD47 is a ubiquitous cell surface protein that is important in immune cell modulation and self-recognition [24]. CD47 is also expressed on tumor cells and may represent a defense against immune cell destruction of cancer cells [87, 88]. Taken together, our results demonstrate that engagement of CD36 or CD47 is sufficient to inhibit NO-driven vascular cell responses, but only CD47 engagement is necessary. CD47 ligation is also sufficient to block the NO-driven stimulation of sGC and cGMP flux in both VSMCs (Fig. 2) and endothelial cells (Fig. 3).

Inhibition of NO/cGMP signaling via CD36

In addition to the CD47 signaling pathway that inhibits NO signaling at the level of sGC and cGK, we recently found an upstream pathway through which TSP1 inhibits eNOS activity [27] (Fig. 4). The TSP1 receptor CD36 is also a fatty acid translocase (reviewed in [25]). Myristic acid was recently shown, in a CD36- and AMP kinase-dependent manner, to activate eNOS [89]. This observation prompted us to examine whether TSP1 and its CD36-binding sequences might modulate NO signaling through antagonizing this function of CD36 and thereby inhibit endogenous NO synthesis. We found that TSP1 and certain CD36-binding TSP1 peptide mimetics are potent inhibitors of myristate uptake into endothelial cells [27]. More than 100 cytoplasmic proteins, including a number of important signaling proteins, are regulated

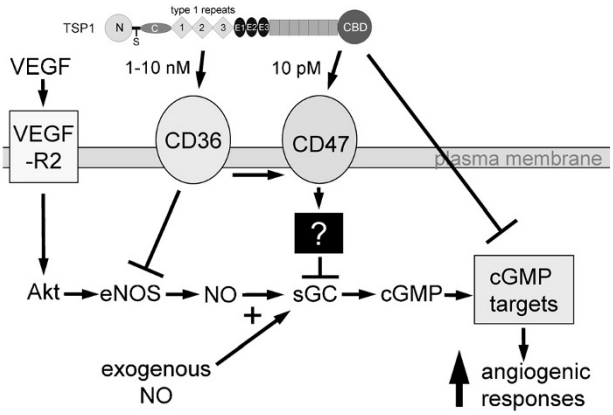

Figure 3. Regulation of NO/cGMP signaling by TSP1 in endothelial cells. VEGF signaling through its receptor activates the kinase Akt, which phosphorylates eNOS on Ser^{1179} [142]. This increases NO synthesis by decreasing the Ca dependence of eNOS. NO activates sGC to increase cGMP synthesis. cGMP acts on several downstream targets in endothelial cells to stimulate pro-angiogenic responses, including survival, growth, motility, and adhesion. At physiological concentrations, TSP1 acts primarily through its receptor CD47 to limit sGC activation. At nanomolar concentrations, TSP1 also signals through CD36 to inhibit the same responses, but CD47 is also necessary for these signals to inhibit cGMP signaling. Therefore, engaging either receptor is sufficient to inhibit NO/cGMP signaling, but CD47 is the necessary receptor of TSP1 action.

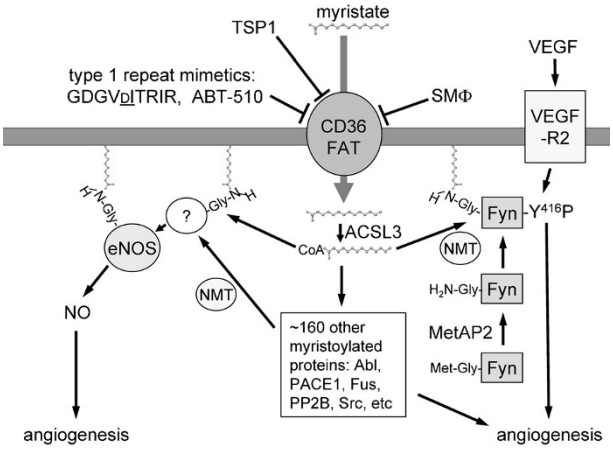

Figure 4. Thrombospondin-1 inhibits angiogenic responses by inhibiting the fatty acid translocase activity of CD36. Several ligands of CD36, including TSP1, a D-Ile derivative of a sequence from its type 1 repeats, the mimetic ABT-510, and the CD36 antibody SMΦ are known to inhibit endothelial cell motility and survival *in vitro* and angiogenesis *in vivo*. These ligands inhibit uptake of fatty acids, including myristate via CD36 [27]. Myristate plays an important role in membrane anchoring of the ~160 proteins in the human genome that contain N-terminal Met-Gly motifs that are substrates for methionine aminopeptidases (MetAP2) and the N-myrisotyltransferases (NMT) that transfer myristate from myristoyl-CoA onto the exposed subterminal Gly residue [90]. Limiting myristate uptake by binding to CD36 prevents membrane anchoring of Fyn and presumably some of the other known or predicted myristoylated proteins. CD36 ligands thereby inhibit myristate- or VEGF-stimulated activation of Fyn assessed by phosphorylation on Tyr^{416}. TSP1 and other CD36 ligands also prevent myristate-stimulated activation of eNOS, presumably by limiting membrane localization and activation of an unidentified myristoylated target [27].

in their membrane association by covalent acylation with myristate at subterminal Gly residues (Fig. 4) [90]. Because activities of eNOS and the Src family kinase Fyn were known to be regulated in a CD36-dependent manner [89, 91], we examined the effect of exogenous myristate and an inhibitory CD36-binding peptide on membrane translocation of these proteins. Although depriving endothelial cells of exogenous myristate had little effect on eNOS translocation, membrane translocation of Fyn was prevented. Adding exogenous myristate or VEGF to endothelial cells restored translocation and functional activation of Fyn, and this was blocked by the TSP1 peptide. Subsequent experiments showed that myristate transport via CD36 controls downstream cGMP signaling via eNOS, and that functional responses of both endothelial and VSMCs stimulated by NO signaling can be inhibited by limiting myristate uptake via CD36. Thus, TSP1 and related anti-angiogenic peptides and antibodies targeting CD36 can limit NO-cGMP signaling via both CD36 and CD47. Therefore, TSP1 can inhibit the NO/cGMP signaling cascade at three different sites (Fig. 3).

TSP1 regulation of NO signaling in platelets

Because TSP1 was first identified as an abundant platelet α-granule component [92], a number of studies have focused on its potential role in blood clotting. Under some conditions, TSP1 promotes platelet aggregation [93], but recombinant domains and peptides derived from TSP1 can either increase or delay platelet aggregation [94–97]. TSP1 also regulates the processing of the platelet adhesion factor von Willebrand factor (vWF) by ADAMTS13 [98], which is associated with increased collagen- and vWF-mediated aggregation of TSP-1 null platelets under static and shear conditions [99]. However, a general physiological role for TSP1 in hemostasis was put in doubt by the failure to find clotting defects in mice with a disrupted *Thbs1* gene, whose platelets lacked any detectable thrombospondins [98, 100].

Our recent work provides a new mechanism to rationalize these conflicting results [22]. *In vitro,* 22 pM TSP1 is sufficient to significantly reverse an NO-stimulated delay in thrombin-driven aggregation under high shear or static conditions. TSP-1 also blocks NO-stimulated cGMP accumulation in platelets. Likewise, recombinant fragments from the type 1 repeats and the C-terminal domain mimic TSP1

inhibition of the NO-stimulated delay in aggregation. In contrast, the recombinant N-terminal domain of TSP1 did not prevent NO-stimulated delay in aggregation and even tended to enhance the NO effect. TSP1-derived peptides known to bind to CD36 and CD47 similarly blocked an NO-driven delay in thrombin-stimulated aggregation.

Endogenous TSP1 also plays a critical role in controlling platelet aggregation. Wild-type platelets demonstrate a modest delay in thrombin-driven aggregation after treatment with exogenous NO. In contrast, NO-treated TSP1 null platelets do not aggregate at all under the same dose of thrombin and require significantly greater amounts of thrombin to obtain the same degree of aggregation as wild-type platelets. This decreased sensitivity to thrombin in TSP1 null platelets was found under both static and high-shear conditions. Consistent with their decreased response to thrombin, TSP1 null platelets have elevated cGMP when exposed to NO or the NOS substrate L-arginine. NO also inhibits platelet adhesion by blocking thrombin-stimulated activation of platelet integrins. Activation of the major platelet integrin αIIb/β3 is regulated by the small GTPase Rap1 [101]. We found that TSP1 modulates this response through blocking NO-driven or direct activation of cGK-I by cGMP [22], which in turn prevents its phosphorylation of the Rap1 GTPase activator Rap1GAP2 on Ser^{7} [102, 103]. Thus, TSP1 increases Rap1 GTP loading, and the active Rap1 activates αIIb/β3 through RIAM and increases platelet adhesion to fibrinogen.

The cytoskeletal regulator VASP is another platelet target of cGK-I, and the ability of NO to delay platelet aggregation requires VASP [104]. Phosphorylation of VASP at Ser^{239} is both a direct and indirect target of NO/cGMP signaling [105], and NO or cGMP-induced phosphorylation of Ser^{239} is inhibited by TSP1 [22]. Therefore, TSP1 opposes the anti-thrombotic activities of NO by promoting both aggregation and adhesion of platelets.

The failure to previously recognize this important role for TSP1 in promoting hemostasis may be an artifact of the conditions under which *in vitro* platelet function is typically studied. Tyrode's buffer contains neither L-arginine nor NO. Hence fresh platelets washed in and suspended in such buffers would become rapidly depleted of their endogenous NO. The lack of difference in clotting in tail-snip assays between wild-type and TSP1 null mice may similarly be explained by the requirement of NO signaling to reveal the role of TSP1 in promoting aggregation. The tail-snip assay allows constitutively generated NO to rapidly escape into the atmosphere, and eNOS null mice similarly show no phenotype in this assay [106]. Under NO-deficient conditions, wild-type and TSP1 null platelets aggregate in comparable fashion and thus would be found to clot with equal rapidity in a tail-snip assay. In a closed system, which is physiologically relevant, endogenously generated NO is known to delay platelet aggregation and adhesion [107–109]. Therefore, the physiological role of TSP1 in hemostasis may be more relevant to internal bleeding and hemorrhage than to superficial bleeding.

TSP1 and wound healing

The TSP1 null mouse developed by Lawler at al. has been a valuable research tool to identify phenotypes associated with the total absence of the protein [100]. TSP1 null mice are viable but tend to produce smaller litters than wild type. In a vivarium with some pathogen exposure, the null mice developed pulmonary infections that led to progressive fibrotic damage in the lungs that is at least partially TGFβ-mediated [110]. Based on its anti-angiogenic activity, TSP1 null mice were expected to show increased rates of wound healing and wound angiogenesis. However, initial reports of wound healing demonstrated delayed healing in the TSP1 null or mice treated with antisense TSP1 oligomers [61, 111] One explanation for this unexpected outcome was that TSP1 stimulates macrophage recruitment, which correlated with a deficit in the chemokine MCP1 in the null wounds [61].

The specific wound model employed may also have influenced the outcome. Dermal punch biopsies were taken from the dorsal cutaneous envelope of the mice. This model is primarily limited by the rate of wound granulation and re-epithelialization, which is only one component of more complex wound healing. Additionally, because these defects were created on the mid-dorsum of mice, which are loose-skinned mammals, wound contracture complicates the measurement of wound healing. The limiting factors then represent the role TSP1 plays in modulating immune cell, keratinocyte, and fibroblast recruitment and functions, which are necessary parts of wound healing. What these experiments do not address, though, is the immediate role of TSP1 in controlling regional blood flow in a wound.

TSP1 and tissue blood flow and survival

Acute trauma and elective surgical procedures result in significant alterations in cutaneous and soft tissue vascular in-flow and tissue perfusion. In many instances, substantial blood flow loss leads to tissue necrosis and increased morbidity and mortality. The ability to maximize soft tissue and cutaneous blood flow has

remained a goal for surgeons. Numerous therapeutic agents have been and continue to be developed for the purpose of selectively increasing blood flow to underperfused tissue units. In several animal models of tissue ischemia, therapy employing L-arginine, cGMP phosphodiesterase inhibitors, or NO donors has increased tissue survival [112–119]. Since we have identified TSP1 as a major physiological regulator of NO-signaling in vascular cells, we investigated whether TSP1 could likewise regulate tissue blood flow.

Our observation that TSP1 inhibits NO-driven VSMC relaxation suggested a role for TSP1 in controlling tissue blood flow. Using blood oxygen level-dependent (BOLD) MRI, which allows non-invasive assessment of tissue blood flow in resting animals, we found that following a NO challenge TSP1 null mice demonstrated a two-fold greater increase in skeletal muscle blood flow compared to wild-type controls [7]. This demonstrates that endogenous TSP1 constantly limits NO signaling to control regional blood flow under resting conditions. These results also demonstrate an immediate role for TSP1 in controlling NO-driven blood flow since the MRI captures tissue alterations in flow in intervals of less than 1 s. Additionally, these findings suggest that regulation of blood flow by TSP1 supersedes the autoregulation of blood vessel response provided by the autonomic nervous system since the studied animals were at rest and with intact autonomic reflexes.

NO-mediated vasodilation plays a critical role in tissue responses to acute ischemia, and hypoxia further increases NO levels through reduction of tissue nitrite [65, 120]. To determine whether the tonic effects of TSP1 in healthy muscle tissue to limit local NO-mediated vasodilation extends to tissue units subjected to a fixed ischemic insult, random dorsal cutaneous flaps were created in wild-type and TSP1 null mice [7]. By definition random cutaneous flaps lack a defined vascular pedicle. Random flaps in which the flap length is twice the flap width typically demonstrate necrosis of the distal 40% of the flap tissue in wild-type animals. However, flaps of identical size in TSP1 null animals demonstrate near complete survival with a mean of only 4% tissue necrosis recorded. Using electron paramagnetic resonance analysis with dermally implanted oxygen-sensitive lithium phthalocyanine probes, we found that improved tissue survival in the TSP1 null flaps correlated with increased tissue oxygen levels. Distal segments of wild-type flaps demonstrated a profound and progressive loss of tissue oxygen from the time of surgery that matched the degree of tissue necrosis. In contrast, TSP1 null flaps, though showing an initial partial drop in tissue oxygen immediately after surgery, showed a progressive increase in tissue oxygen to levels comparable to unoperated cutaneous units by 72 h. Therefore, TSP1 is limiting for tissue blood flow and survival of fixed soft-tissue ischemia. Parallel experiments demonstrated enhanced tissue blood flow and survival to a fixed ischemic insult in CD47 null animals [121]. In contrast, random cutaneous flaps in CD36 nulls underwent a degree of tissue necrosis equal to or slightly greater than that found in wild-type control animals [121]. Therefore, CD47 is the critical TSP1 receptor for regulating blood flow under ischemic stress.

Tissue survival was also found to be limited by TSP1 through CD47 in a complex composite tissue ischemia model [121]. Blood inflow to the mouse hindlimb is minimally redundant. The femoral artery represents over 90% of the inflow to the limb. Upon ligation of the femoral artery at the level of the inguinal ligament, the mouse hindlimb experiences profound and near total hypoxia. Post-ligation, rapid vascular remodeling of existing collaterals only partially restores limb perfusion. Thus, femoral arterial ligation results in significant tissue necrosis in wild-type animals, often manifesting as gangrene of the toes and ulceration of the skin and underlying muscle. However, similar interruption of blood flow to the hind limbs of TSP1 and CD47 null mice resulted in complete limb survival without any cutaneous or muscle tissue necrosis or acral part gangrene. Even more startling, the null hind limbs demonstrated immediate and significant vascular remodeling and dilation of existing collateral vessels, detectable by laser Doppler imaging within minutes of arterial ligation (Fig. 5). Wild-type hind limbs showed minimal vascular remodeling immediately following vessel ligation. The increased tissue survival and vascular remodeling detected within minutes in TSP1 and CD47 null animals correlated with increased return of limb blood flow and tissue perfusion.

The above results demonstrate that endogenous TSP1, via its receptor CD47, limits tissue survival of a fixed ischemic insult and improved restoration of blood flow. These findings have clinical relevance to certain ischemic injuries and to surgical flaps where vascular connections are maintained through the pedicle [122]. However, we were interested in whether TSP1 also plays a role in tissue survival of ischemic injury where such vascular connections are absent. Such conditions are regularly encountered in skin grafting [122]. By definition a skin graft is initially without any circulation. The nutrient and oxygen needs of the skin graft are met through diffusion from the underlying wound bed to which it is applied. After an interval of 3–10 days, a new circulation is established, and the graft then receives nutrition via this circulation. Although split-thickness skin grafts have

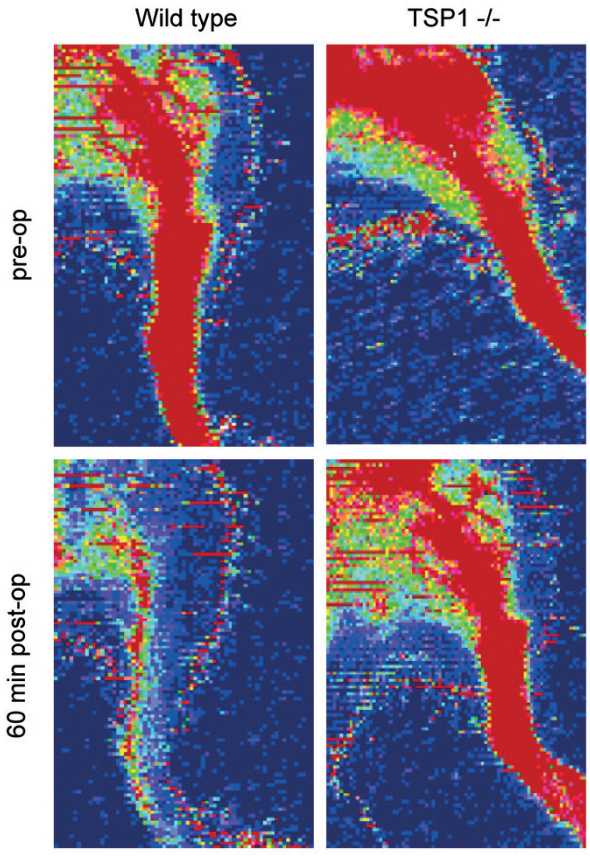

Figure 5. Acute effects of endogenous TSP1 on hind limb perfusion following ischemic injury. Wild-type and TSP1 null mice underwent ligation of the femoral artery at the level of the inguinal ligament, and limb perfusion was measured during the first post-operative hour. An increased persistence of limb perfusion is demonstrated in the TSP1 null limb.

broad clinical use, full-thickness grafts in humans have very limited success due to their limited revascularization. Full thickness skin grafts (FTSGs) in wild-type mice similarly went on to near complete necrosis within 3–4 days, but remarkably, FTSGs in TSP1and CD47 null animals demonstrated essentially complete healing [123].

Cross-transplantation between wild-type C57BL/6 mice and mice lacking TSP1 or CD47 demonstrated that graft survival is primarily determined by the genotype of the wound bed [123]. Wild-type grafts placed on TSP1 or CD47 null wound beds always showed enhanced tissue survival. In contrast, null grafts placed on wild-type wound beds underwent increased necrosis. Perfusion analysis via laser Doppler imaging found a direct correlation of graft survival with tissue blood flow. Thus, even in this full ischemia model TSP1 limits tissue blood flow, vascular remodeling and tissue survival.

TSP1 and blood flow in aged animals

It is clear that TSP1, via CD47, limits the ability of tissue to respond to ischemic stress and controls NO-driven alterations in blood flow. These findings in young animals, though of profound significance for people undergoing surgery or suffering from soft tissue trauma, do not necessarily extend to elderly patients who have advanced vascular pathology, including peripheral vascular disease and atherosclerosis. Endogenous NO production and eNOS expression in blood vessels markedly decreases with aging [124, 125]. Vascular TSP1 expression may also change with age. Decreased TSP1 expression was reported in Bruch's membrane and choroidal vessels in eyes of patients with age-related macular degeneration [126]. Conversely, increased TSP1 has been reported in ischemic limbs of aging diabetics [2], in the glomeruli and tubulointerstitium of the kidney in aging rats [127], and in atherosclerotic vessel walls [3, 4]. The related protein TSP2 was also found to increase with aging in skin in mice and was associated with delayed wound repair [128, 129].

Wild-type mice aged to between 14 and 18 months demonstrated even greater degrees of tissue necrosis to an ischemic insult than young animals, with some animals experiencing complete flap loss [130]. TSP1 and CD47 null animals subjected to the same ischemic insult showed near complete tissue survival in the face of advanced age. Quite startling, NO-driven increases in skeletal muscle blood flow in old TSP1 null animals were greater than similar results obtained in young wild-type animals. In the absence of TSP1, the vascular response to NO remains more dynamic and confers a performance advantage despite advanced age compared to young animals. Even more interesting, in apoE null mice, which acquire diet-induced atherosclerotic vascular disease [131], ischemic tissue survival was significantly enhanced through suppressing CD47 in the tissue unit [130].

Therapeutic enhancement of ischemic tissue survival by targeting TSP1 or CD47

A significant number of individuals over the age of 65 will develop some degree of vascular disease with attendant alterations in tissue perfusion [132–135]. The ability to selectively enhance tissue perfusion would be of great therapeutic benefit. Attempts with present agents have met with limited success. The dominant role NO plays in regulating blood vessel diameter suggested that targeting TSP1/CD47 could potentiate the ability of endogenous NO to enhance blood flow and vascular remodeling. Such an ap-

proach could synergize with current therapies that provide an exogenous NO source to increase blood flow. Wild-type cutaneous flaps treated with a monoclonal antibody that recognizes murine TSP1 demonstrated increased tissue survival [121]. The same TSP1 antibody enhanced tissue preservation in wild-type ischemic hind limbs. In all instances, the therapeutic was suspended in normal saline and delivered by direct injection into the target tissue unit.

Tissue survival could also be increased by targeting the TSP1 receptor CD47. One approach employed the anti-murine CD47 antibody 301 to prevent TSP1 signaling through CD47. This antibody prevented tissue necrosis in cutaneous flaps of wild-type mice and in FTSGs [121, 123].

The second approach was to temporarily suppress CD47 expression at the site of injury. An antisense morpholino oligonucleotide complementary to the 5′ region of murine and human CD47 mRNA was designed and verified by Western blotting to prevent translation of this mRNA in vascular cells *in vitro* [121]. The morpholino was directly injected into ischemic wild-type tissues with dramatic increases in tissue survival. This same morpholino was verified to significantly suppress CD47 expression in treated tissue flaps by immunohistology. The morpholino was also effective in FTSGs [123].

Future directions

Understanding the antagonism of NO/cGMP signaling by TSP1 and our developing ability to therapeutically modulate this process may have implications for a wide range of diseases. In healthy tissue, physiological TSP1 levels tonically limit NO signaling (Fig. 6). We propose that this role of TSP1 is important for hemostasis and acute survival of traumatic injuries. However, we have also shown in several acute ischemic injury models that elevated TSP1 expression effectively ablates the ability of NO to maintain or restore tissue perfusion. Our work also indicates that the elevated TSP1 characteristic of aging tissues can exert chronic effects to limit NO signaling. This paradigm needs to be tested in other chronic diseases. Peripheral vascular disease underlies a host of chronic disease states including diabetes, atherosclerosis, ischemic heart disease, stroke, and dementia, varying degrees of which ultimately affect a majority of individuals over the age of 65. Decreased production of endogenous NO and increased expression of TSP1 are common features of blood vessels in these patients. Then targeting TSP1/CD47 could have a substantial therapeutic effect by maximizing the ability of NO to improve blood flow and tissue perfusion.

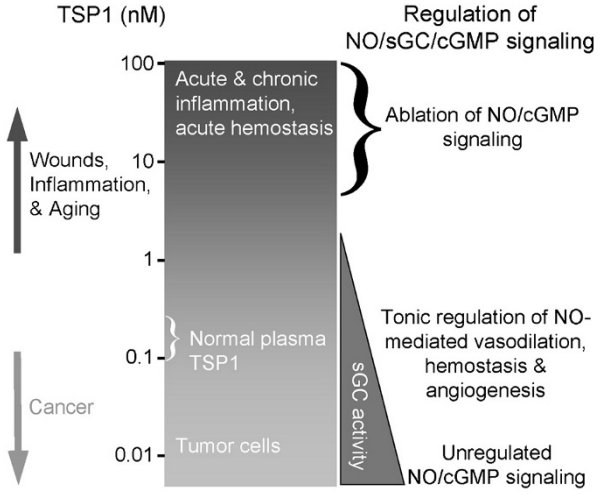

Figure 6. Concentration-dependent control of NO/cGMP signaling by TSP1. Physiological levels of TSP1 in tissues and blood (100–200 pM) are sufficient to tonically limit NO/cGMP signaling. Hemostasis, wounding, and aging are known acutely or chronically to increase TSP1 to levels sufficient to prevent all activation of sGC. Conversely, malignant progression is frequently associated with loss of TSP1 expression in tumor cells, which may relieve negative regulation of NO/cGMP signaling in tumor cells. However, stroma in some tumors also exhibit elevated TSP1 expression, which may limit NO signaling in the tumor vasculature and infiltrating immune cells as well as suppress angiogenesis in distant metastases [49].

Contrary to these diseases, many cancers are characterized by loss of TSP1 expression. Based on our paradigm, this should remove the tonic inhibition of NO signaling by TSP1 (Fig. 6). Thus we predict that cancers may enjoy enhanced blood flow due to unregulated vasodilation of their blood supply. Based on this concept, we should also seek therapeutic strategies to restore such regulation and thereby limit the perfusion and angiogenic responses needed to sustain tumor survival and growth.

A key long-term objective of this work is to translate these findings into therapeutic applications. Murine cutaneous vascular anatomy is quite different from that of humans, so pigs are often used for preclinical testing of therapies targeting these tissues. Our ongoing work indicates that the CD47 morpholino can also enhance ischemic tissue survival in a porcine model. Therefore, to date we have shown that targeting of TSP or CD47 can block NO signaling in murine, bovine, porcine, and human vascular cells, and in at least two of these species this inhibition can enhance ischemic tissue survival. Pending pharmacokinetic and toxicology evaluation, we hope that these agents can progress to clinical testing.

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