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Review

The role of thrombospondin-1 in apoptosis

P. Friedl[a], P. Vischer[b] and M. A. Freyberg[c,*]

[a] Technical University of Darmstadt, Inst. Biochem., Petersenstr. 22, 64827 Darmstadt (Germany)
[b] University of Münster, Inst. Arteriosklerosеforschung, Domagstr. 3, 48149 Münster (Germany)
[c] CytoTools GmbH, Petersenstr. 22, 64827 Darmstadt (Germany), Fax + 49 6151 166025, e-mail: freyberg@cytotools.de

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Abstract. The thrombospondins are a family of extracellular proteins that participate in cell-to-cell and cell-to-matrix communication. They regulate cellular phenotype during tissue genesis and repair. Five family members, each representing a separate gene product, probably exist in most vertebrate species. Like most extracellular proteins, the thrombospondins are composed of several structural domains that are responsible for the numerous biological functions that have been described for this protein family. Considerable progress has been made towards understanding the function of thrombospondins. The role of thrombospondin in the process of apoptosis or programmed cell death has recently come into focus. In this review we will concentrate on the role of thrombospondin-1 in the broad field of apoptotis research.

Key words. Thrombospondin; apoptosis; integrin; CD36; CD47.

Introduction

The extracellular matrix is not only an environment which provides support for cells and tissue but also a source of complex information that controls several important cellular functions, such as cell shape, migration, adhesion, cell growth, differentiation and survival. These activities are regulated by cell receptors expressed at the cell surface communicating with the extracellular matrix, composed of collagens, proteoglycans and glycoproteins. Paul Bornstein used the term 'matricellular protein' [1] to differentiate between structural proteins of the extracellular matrix and proteins without a direct structural role but which modulate cell matrix interaction and other cellular responses. These properties are shared by a group of unrelated but functionally similar glycoproteins including SPARC (secreted protein acidic and rich in cysteine), tenascin C, osteopontin and thrombospondins (TSPs) [2 and references cited].

TSPs are a family of five known proteins with modular and multidomain structures. The first member of this gene family that was identified and later designated asTSP-1 was first found to be a constituent of the α granules of platelets, where it is involved in platelet aggregation and clot formation [3, 4]. Initial investigations concentrated on the role of TSP-1 during platelet aggregation and fibrin clot formation. Later observation of the protein's synthesis and secretion into the extracellular matrix of this protein by a variety of cells in vitro and its presence as a component of the extracellular matrix in solid tissue pointed to a more general function of TSP in cellular processes [5]. These include cell attachment, motility, proliferation and influence on angiogenesis and tumor progression [6–10].

In the last 10 years it became apparent that another four proteins related to TSP-1 were also encoded by homologous but unlinked genes. They were termed TSP-2, TSP-3, TSP-4 and TSP-5/COMP (cartilage oligomeric matrix protein) [11]. Given the high concentration of TSP-1 in blood platelets and its established role in purification, the

* Corresponding author.

most detailed studies of structure and cellular function have been undertaken on TSP-1.

TSP-2 is closely related in structure to TSP-1 but differs in its tissue distribution and from the distinct phenotypes of TSP-1 and TSP-2 null mice [12–15]. TSP-1 and TSP-2 are homotrimeric proteins consisting of three identical protein chains. TSP-3, TSP-4 and COMP/TSP-5 are pentameric proteins lacking a procollagen domain and three type 1 repeats found in TSP-1 and TSP-2. The members of the TSP family have been divided into two subgroups based on these structural differences.

Several excellent reviews have already been published on TSPs [16–18]. We will therefore concentrate only on TSP-1, describing some structural and ligand binding properties and will focus on its function in the process of apoptosis.

Structure of TSP-1

The gene for TSP-1 is located in band q15 on human chromosome 15 and encodes a homotrimeric, cysteine-rich, glycosylated and β-hydroxylated protein of about 450 kDa. Besides N-glycosylation of TSP-1, which has been described for platelet TSP-1 and newly synthesized TSP-1 in endothelial cells, an additional posttranslational modification of TSP-1 was recently observed [19, 20]. This modification involves the C-mannosylation of tryptophan residues and a novel O-fucosylation [21].

Like other extracellular matrix proteins, TSP-1 possesses a modular and multidomain structure (fig. 1). At the N-terminal end, each subunit contains a small globular domain, with heparin-binding properties, followed by a short linear region with two cysteines that form interchain disulfide bonds [10]. The following module of a length of about 90 amino acids is homologous to procollagen. The next domain, comprised of three type 1 repeats, is present only in TSP-1 and TSP-2. These repeats of about 60 amino acids represent a module which is widely distributed in vertebrates [11]. It is found in the complementary factors properdin and C6–C9 as well as in malaria circumsporozoite proteins [22]. The F-spondin family and the ADAMTS (a disintegrin and metalloprotease with TSP motifs) family are further members of this group of proteins containing up to seven type 1 repeats [23, 24]. Together, these proteins are described as members of the thrombospondin repeat (TSR) superfamily [25].

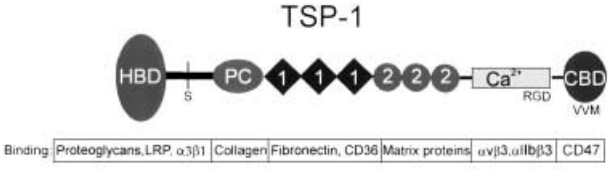


Figure 1. The structure of TSP-1. The TSP-1 peptide chain consists of (N-terminal) a heparin binding domain (HBD) that binds to cell surface proteoglycans, lipoprotein-receptor related protein (LRP) and β1 integrins; a domain related to the N-terminal peptide of collagens (PC), the collagen binding domain ; three type 1 repeats that contain binding sites for fibronectin and CD36; three type 2 repeats that are responsible for the interactions with soluble and matrix proteins; a repetitive (seven repeats) Ca^{2+}-binding region that contains a single RGD sequence that binds to β3 integrins; the C-terminal cell binding domain (CBD), which contains the VVM sequences that bind to CD47.

Adjacent to the TSRs, each subunit contains three type 2 repeats, which are structurally homologous to epidermal growth factor. This module is present in all members of the TSP family and is also a constituent of other adhesive glycoproteins such as fibronectin. TSP-1 is a calcium-sensitive glycoprotein. Seven type 3 repeats form the primary calcium binding domain [26]. Measurement of direct binding of calcium reveals that each TSP subunit binds 11 or 12 calcium ions of which up to 10 calcium ions are bound in the aspartate-rich type 3 repeats [27]. The presence of calcium influences the structure and function of TSP-1. Treatment of the protein with EDTA leads to an unravelling of the C-terminal globular domains, whereas the central stalklike region of the molecule, containing the procollagen to type 3 modules, enlarges [28]. The presence or absence of calcium also modulates the sensitivity of TSP-1 to fragmentation by proteases. Within the last of the type 3 repeats each TSP-1 subunit contains an arginine-glycine-aspartic acid (RGD) sequence, a known recognition motif for integrins. Its ability to modulate adhesion is affected by the calcium concentration and position of a free sulfhydryl group found in this domain which alter the accessibility of this site [29].

Another globular domain of about 260 amino acids is present at the C-terminal end (CBD). Presence in this single domain is common to all the members of the TSP family, while the amino-terminal globular domain is the most variable part of the molecule for all (fig. 1).

Ligands and ligand recognition

Because of its modular structure TSP-1 is able to interact with a wide variety of macromolecules and cell surface structures. Interaction with structural proteins found in the extracellular matrix such as different collagens, laminins and fibronectin might modulate the functional behavior of those proteins while altering TSP-1 activity. This has been observed in melanoma cells where binding of fibronectin strongly enhanced the adhesive activity of immobilized TSP-1 [30].

Binding to several proteases, including thrombin, plasmin, cathepsin G or matrix metalloproteinase 2 have been reported [31, 32]. TSP-1 modulates directly or indirectly several biological processes.

The interaction of TSP-1 and latent transforming growth factor-β1 (TGF-β1) has been shown to be of physiological relevance. TGF-β1 exerts a wide range of biological effects through interaction with high-affinity TGF-β1 receptors [33]. TGF-β is produced in a latent form consisting of the mature TGF-β dimer and a noncovalently related latency-associated peptide (LAP). Latent TGF-β is activated through limited proteolysis by plasmin or through a nonproteolytic activation by TSP-1 [34, 35]. Murphy-Ullrich's group has shown that activation by TSP-1 requires a WXXW motif to be present in each of the three type 1 repeats for binding to the mature TGF-β1 dimer and a proposed second recognition sequence of KRFK to bind to a conserved sequence (LSKL) near the amino-terminus of LAP [36]. Some recent studies on TSP-1-deficient platelets and on smooth muscle cell cultures have suggested that in addition to the described TSP-1 mediated activation, other TGF-β activation mechanisms also exist [37, 38].

Part of the observed biological effect of TSP-1 requires the direct binding of the molecule to specific cell surface receptors. The binding to different members of cell surface molecules has been reported to include CD36, integrins, CD47 or integrin-associated protein, the low-density lipoprotein receptor-related protein, a 105/80-kDa heterodimeric membrane protein, proteoglycans and sulfatides.

CD36 is a transmembrane protein with collagen-binding properties and a scavenger receptor for modified low-density lipoprotein (LDL) [39]. Interaction with TSP-1 was observed on platelets, monocytes and endothelial cells [40–42]. The CSVTCG motif of the second and third type 1 repeats has been identified as the CD36 high-affinity binding site [43]. Using antibodies against CD36 or fusion proteins containing the TSP-1 binding site inhibited the effects of TSP-1 on endothelial cells [44]. In lung carcinoma cells, another receptor not identical to CD36 is also able to recognize the CSVTCG sequence in TSP-1 [45]. Dawson et al. have shown that a sequence other than CSVTCG also mediates TSP binding to CD36 [46].

Several integrins have been described as ligands for TSP-1. The arginine-glycine-aspartic acid (RGD) sequence is the main recognition site for nearly half of the known integrins [47]. Other integrins bind related sequences in their ligands. TSP-1 contains an RGD integrin recognition site in the seventh type 3 repeat. This motif binds cells like endothelial and smooth muscle cells expressing αVβ3 integrin. This interaction is controlled by disulfide interchange events in the calcium binding region of TSP-1 [48, 49]. On activated T lymphocytes cell attachment involves α4β1 and α5β1 integrins, although the precise binding sites for TSP-1 have not been determined for their interaction [50]. The participation of α3ß1 integrin in the interaction of TSP-1 with breast carcinoma cells (MDA-MB-435 and MDA-MB-231) has more recently been identified. This integrin is partially inactive but can be activated with serum, insulin, insulin-like growth factor or ligation with CD98 [51]. Observation of another regulative activity of fibronectin provided confirmation. Exogenous fibronectin enhanced the ability of TSP-1 to be recognized by α3β1 integrin [30]. A sequence in the N-terminal globular region, containing residues 190–201, has been identified as the recognition sequence and is found in all mammalian TSP-1 sequences [52].

CD47 or IAP (integrin-associated protein) is a 50-kDa transmembrane glycoprotein expressed in a large number of mammalian cells [53]. It consists of a highly glycosylated extracellular immunoglobulin-like domain, a five-membrane-spanning region, and a short cytoplasmatic tail. IAP can associate with some integrins in a supramolecular complex, including the RGD-dependent αVβ3, the platelet fibrinogen receptor αIIbβ3 and the collagen receptor α2β1. Additional ligation of TSP-1 with this complex stimulates the activation state of the integrins, affecting several biological functions on melanoma cells, platelets and smooth muscle cells [54–56]. Responsible for the interaction of TSP-1 with IAP are two sequences within the C-terminal globular domain of the molecule [57]. Both contain the adhesive motif Val-Val-Met (VVM). Affinity chromatography with two active peptides, 7N3-1 (RVVM) and 4N1K (RFYVVMWK), facilitated identification of IAP as a receptor for this cell attachment site [58]. Complexing of TSP-1 with IAP/integrins initiates intracellular signalling events, which are induced through the direct association of CD47 with heterotrimeric G proteins of the Gi family [59].

Heparin binding was one of the early features observed while characterizing TSP-1. Today several binding sites for heparin, proteoglycans and sulfated glycolipids have been identified. The amino-terminal globular domain represents the major heparin binding site. Two sequences rich in basic residues are necessary for high-affinity heparin binding in vitro [60]. TSP-1 contains heparin-binding sequences in the type 1 repeats as well. The motif Trp-Ser-Pro-Trp (WSPW) was identified as the minimal active sequence for heparin or sulfatide binding [61]. A peptide containing this motif together with a flanking sequence of basic amino acids supported cell adhesion and triggered intracellular signals that modulated the function of αvβ3 integrin on melanoma cells [62]. However, recent investigations comparing the interaction of heparin with intact TSP-1 or heparin-binding fragments of TSP-1 revealed that the major interaction between TSP-1 and heparin is mediated by the amino-terminal domain [63].

In vivo, it is mainly heparan sulfate proteoglycans (HSPGs) that assume the functional role of heparin. In several studies the receptor-like function of HSPGs has been demonstrated. Investigation of endothelial cells or CHO cells has shown that during TSP-1 metabolism, the

binding and endocytosis process of TSP-1 is mediated by heparan sulfate glycosaminoglycans and can be inhibited by heparin, heparitinase treatment or modulation of the heparan sulfate chains [64–67]. Studies using gold beads coated with TSP-1 fragments demonstrated that the N-terminal heparin binding domain is an essential structural attribute for the binding and degradation process [68]. It also appears that binding and degradation of TSP-1 involves the LDL receptor-related protein (LRP) in addition to heparan sulfate proteoglycans [69]. But the exact interrelationship between heparan sulfate proteoglycans and LRP during this binding and degradation process is not fully elucidated.

The structural requirements of heparan sulfate which mediate the binding activity of TSP-1 have recently been determined [70]. Endothelial cell-derived HS chains are composed of domains of noncontiguously arranged highly sulfated disaccharides separated by extended sequences containing predominantly N-acetylated sequences of low sulfation. Only specific domains of deca- to tetradecasaccharides within these HS chains possessed binding affinity to TSP-1.

As well as the cellular binding properties and degradation of TSP-1, a novel role for cell surface HSPGs was also found. It was shown that the membrane-spanning syndecan 1 is necessary for spreading and fascin spike formation by C2C12 myoblastic cells on TSP-1 [71].

TSP-1 also interacts with other glycosaminoglycan structures. This was demonstrated for decorin, a small leucine-rich proteoglycan composed of a 40-kDa protein core substituted by a single-tissue-specific chondroitin/dermatan sulfate chain. Decorin, which delays and weakens adhesion of skin, possesses high-affinity binding properties to TSP-1 [72]. These binding properties involve the glycosaminoglycan chain as well as the core protein. Adhesion studies with fusion protein and peptides provided evidence that decorin inhibits adhesion to TSP-1 by interacting with a KKTR motif within the N-terminal globular domain [73]. In a previously mentioned investigation on the role of decorin in angiogenesis, a modulating influence of TSP-1 on the inhibition of tube formation by decorin was observed [74].

TSP-1 not only supports cell adhesion but can also reduce focal adhesions in spread cells [75]. This focal adhesion/reorganizing activity is localized in a 19-amino acid sequence (termed hep I peptide) in the N-terminal heparin binding domain of TSP-1 [76]. The binding affinity of this motif to heparin points to heparan sulfate proteoglycans as the receptor protein. But further studies have revealed that calreticulin, a 60-kDa calcium-binding protein on the cell surface of endothelial cells mediates focal adhesion disassembly by the hep I motif of TSP-1 [77].

Other cell surface structures described as TSP-1-binding molecules include phosphatidylserine, sulfated glycolipids or sulfatides and have been described as binding partners in melanoma cells and red blood cells. In sickle cell disease the abnormal adherence of red sickle blood cells (SS RBC) to the vascular endothelium plays an important role in the overall vaso-oclusion process [78]. Although many mechanisms have been implicated in the binding of SS RBC to endothelium, mounting data suggest the importance of TSP-1 in this process. The adhesive protein TSP-1 promotes SS RBC adhesion to cultured endothelium serving as a bridging molecule between endothelial and SS RBC receptors. In vitro studies under static or flow conditions identified sulfated glycolipids, phosphatidylserine and CD47 as adhesion receptors on SS RBCs for TSP-1 [79–81].

TSP-1 and its function in apoptosis

Apoptosis is a process of programmed cell death that was initially defined by morphological characteristics, including cell shrinkage, membrane blebbing, chromatin condensation and nuclear fragmentation [82]. Apoptosis is a physiological process for the depletion of unwanted or senescent cells without inflammation [83]. Apoptosis works simultaneously but opposite to mitosis in counterbalancing and regulating tissue kinetics [84]. The general importance of apoptosis has been widely recognized only in the past decade, and its significance is currently being evaluated in many areas of biology. Apoptosis is controlled by a conserved program and acts in embryonic development and tissue homeostasis and can also be induced by various pathological stresses. Apoptosis to both excessive and lesser degrees may have pathological implications [85, 86]. In the second part of this review we want to focus on the role of TSP-1 in apoptotic processes.

Cell death in immune cells within the context of TSP-1

Recognition of apoptotic cells

Among the immune cells, apoptosis and TSP-1 have a key function in recognition and phagocytosis of cells undergoing programmed cell death. In vivo, the normal fate of cells undergoing apoptosis is recognition, uptake and degradation of the dying cell by phagocytes. Apoptotic cells are marked for disposal by mechanisms which are still poorly understood, but there is initial evidence from in vitro experiments that the phagocyte receptors, including the TSP receptors (integrin $\alpha V\beta 3$, CD36), and the scavenger receptors are involved [87]. TSP receptors were first detected in relation to immune cells by their implication in recognition of apoptotic neutrophils by human monocyte-derived macrophages [88]. TSP was present in the interaction, in both association with the macrophages and in solution. The data indicated that in-

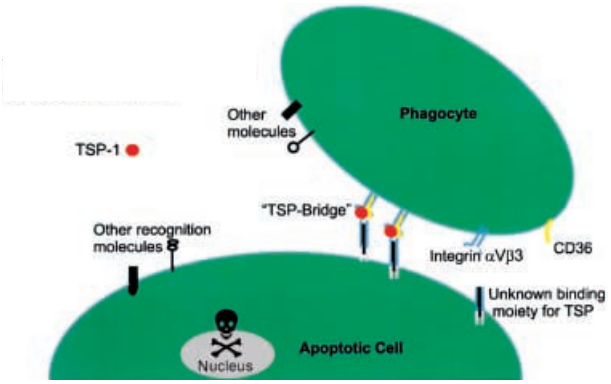


Figure 2. TSP-1 and the recognition of apoptotic cells. There are different molecules implicated in phagocyte recognition of apoptotic cells. Thrombospondin receptors, CD36 (yellow) and the $\alpha V\beta 3$-integrin (blue), are involved in recognition of apoptotic neutrophils (eosinophils and lymphocytes) by macrophages. TSP-1 (red) has a 'bridging' function in this process [84].

tegrin $\alpha V\beta 3$ and CD36 cooperated in recognition of apoptotic cells on monocyte-derived macrophages, binding ('bridging') TSP1 by a two-point mechanism (fig. 2). The TSP-1-$\alpha V\beta 3$ interaction could be inhibited by Arg-Gly-Glu (RGD)-bearing peptides or antibodies to TSP1 and $\alpha V\beta 3$ [87–89]. Specific inhibition of $\alpha V\beta 3$, CD36 and TSP1 by RGD peptide and monoclonal antibodies (mAbs) indicates that the same mechanism is used by monocyte-derived macrophages in recognition of apoptotic eosinophils and lymphocytes [90, 91].

One exception to this complete bridging complex was found on human glomerular mesangial cells. There was no expression of CD36 and no evidence for the involvement of alternative phagocyte receptors of TSP1. Specific inhibition of the vitronectin receptor, integrin, by RGD peptide and mAbs indicated, however, that mesangial cell phagocytosis of apoptotic cells also involves the $\alpha V\beta 3$/TSP1 mechanism [92]. These results point out the central role of TSP1 in the process of recognition and phagocytosis of apoptotic cells, although the entire mechanism still requires further investigation. In contrast, there are examples of TSP1-independent adherence and recognition from leukocytes to apoptotic endothelial cells [93].

Induction of apoptosis in immune cells

Apoptosis can be induced in human monocyte-derived macrophages by oxidized LDL. This process involves CD36 and the activation of caspase-3. TSP inhibits the induction of apoptosis by OxLDL on a dose-dependent basis [94]. TSP binds to a region of CD36 in close proximity to one of the two proposed binding domains for OxLDL. These results suggest that binding of OxLDL to CD36 initiates apoptosis of human macrophages. TSP itself did not, in this case, induce apoptosis in human macrophages.

CD47, another receptor molecule for TSP-1, has been identified as a mechanism of cell death in B cell chronic lymphatic leukemia clones. Immobilized TSP-1 or immobilized monoclonal antibodies against CD47 induced features of apoptosis in all clones (cell shrinkage, loss in mitochondria membrane potential and exposure of phosphatidylserine) without nuclear characteristics and caspase activation. Cross-linking of CD47 was absolutely required in this instance of apoptosis induction. TSP-mediated killing was dependent on CD47 binding, because the use of the 4N1K peptide, corresponding to the CD47 binding site of TSP, excluded other possible TSP receptors (CD51/CD61) [95].

In conclusion, it can be said that TSP-1 plays an important physiological role in the clearance of immune cells by acting as a molecular bridge between phagocytic and apoptotic cells through interactions of TSP with $\alpha V\beta 3$/CD36 receptors on these cells (fig. 2).

Results from these studies of apoptosis induction suggest a new strategy for the development of alternative therapeutics in pathological conditions like cancer.

TSP-1 and the induction of apoptosis in angiogenesis

The first time TSP was directly connected to the induction of apoptosis was in a publication dealing with cancer research in 1997 [96]. In the field of cancer biology TSP had been discussed thus – far only as a modulator of cell adhesion, motility and growth (angiogenesis). Guo et al. showed that type I repeats of TSP1 or the intact glycoprotein induced programmed cell death in bovine aortic endothelial cells [96]. Induction of DNA fragmentation by the peptides was decreased when endothelial cell cultures reached confluence. This implies that induction of apoptosis by TSP1 analogs is not generally cytotoxic and depends upon a lack of strong survival-promoting signals such as those provided, for example, by a fibronectin matrix. So the ability of TSP1 or the peptide analogs to inhibit growth and induce apoptosis is dependent on other external signals. The mechanism and the connection to the physiological situation in the organism have not yet been described.

The biological function of TSP in the process of angiogenesis has been discussed since 1990 [97]. TSP-1 was the first naturally occurring inhibitor of angiogenesis discovered. Loss of TSP-1 is crucial for the angiogenic switch in many tumor models. Angiogenesis is a process of capillary formation from preexisting blood vessels. It is tightly controlled by the balance between positive and negative environmental signals. Complete supression of angiogenesis in normal adult tissues is only lifted in response to a local demand for additional blood flow such

as during wound healing or in reproductive events. By contrast, in pathological situations, angiogenesis may be initiated by ischemia or by a rapid increase of tissue cells such as tumor growth. The effect of TSP-1 on angiogenesis is a receptor-mediated event. TSP-1 is unable to block endothelial cell migration if it cannot bind to the CD36 receptor molecule [44]. The signaling pathway downstream from CD36/TSP-1 complexes involves recruitment and activation of the Src-related kinase, $p59^{fyn}$ and consecutive activation of the stress-activated kinase, p38MAPK. Activation of p38 is Fyn dependent and requires caspase-3 as in proteolytic activity [98]. The presence of caspases points to an apoptotic pathway. The conclusion we are led to is that TSP-1 prevents the formation of new blood vessels by inducing apoptosis in the activated endothelial cells [99].

Further investigation has shown that TSP-1 induces endothelial cell apoptosis and inhibits angiogenesis by activating the caspase death pathway [100]. TSP-1 mediates endothelial apoptosis in association with increased expression of the proapoptotic protein, Bax, decreased expression of the anti-apoptotic protein Bcl-2 and activation of caspase-3. TSP-1-induced apoptosis is dose-dependent [100]. Transfected cell lines have also shown that cells expressing the full-length TSP-1 or the type 1 repeats both mediate the induction of endothelial apoptosis. These findings suggest that CD36, the binding partner of the type I repeats, is sufficient for mediating angiostatic effects through induction of endothelial apoptosis. This idea was established by Jimenez et al. It was shown that the inhibition of angiogenesis both in vitro and in vivo as well as the induction of apoptosis by TSP-1 require activation of CD 36, $p59^{fyn}$, caspase-3 like proteases and p38 mitogen-activated protein kinases [101]. Here again, these findings indicated that TSP-1 acts in vivo by inducing receptor-mediated apoptosis in activated endothelial cells. Fragments of TSP-1 lacking integrin binding motifs were also able to induce apoptosis and to inhibit angiogenesis, thus demonstrating independence from integrin signalling. In detailed studies, Jimenez et al. found that c-Jun N-terminal kinase activation is required for the inhibition of neovascularization by TSP-1 [102]. Thus two stress-activated kinases, JNK-1 and p38 MAPK, seem to be integral parts of the signalling network that leads from CD36 to the apoptosis-dependent inhibition of angiogenesis by TSP-1.

Further examination in the field of cancer research showed that overexpression of TSP-1 inhibited the tumor growth of xenotransplants and completely abolished tumor formation [103]. The effects of TSP-1 on tumor cell growth were indirect since tumor cell proliferation rates both in vivo and in vitro as well as susceptibility to induction of apoptosis by serum withdrawl were unchanged in tumor cells overexpressing TSP-1. This overexpression upregulated CD36, the TSP-1 receptor, leading to enhanced adhesion of cells to TSP-1. The exact biological function of TSP-1 and the role of apoptosis in the context of tumor cells must still be investigated.

TSP-1 as mechanosensitive death mediator

Cell growth, differentiation and survival responses are the result of the integration of numerous chemical and biophysical cues from the cells' surrounding environment. TSP-1 expression is highly regulated during development and following cellular injury. As an extracellular protein, TSP-1 modulates focal adhesions in endothelial cells [75]. TSP-1 stimulates the reorganization of actin stress fibers and the disassembly of focal adhesion complexes, but has only a minimal effect on cell shape. The link between the actin stress fibers and the submembranous focal adhesion plaque is effectively disrupted without visibly affecting the integrin-extracellular matrix protein link [104]. Anchorage-dependent cells require cell adhesion for survival. Adhesion-dependent cell death is termed 'anoikis' and it is a mechanism for preventing cell growth at inappropiate locations and for cavitation during embryogenesis. Cell shape and an extended spread morphology are essential for survival [105]. Cell attachment and spreading involve the activation of focal adhesion kinases and phosphatidylinositol 3-kinase (PI3K), and both of these mediators may act in anti-apoptotic signalling [106].

In 1997, it was reported for the first time that lack of hemodynamic forces induces apoptosis in endothelial cells [107]. Three years later the molecular background by which mechanical stimulus and apoptosis are coupled was identified as an autocrine loop of TSP-1 with the $\alpha V\beta 3$ integrin/IAP (CD47) complex as its receptor [108] (fig. 3). In contrast to common apoptosis inducers, the lack of hemodynamic force initiates a low basal level of apoptosis only. It steadily increases with time, however, preventing complete vessel destruction given only a transient offset of flow. Vascular endothelial cells secrete TSP-1 and express CD47 in postconfluent static monolayers only and not under flow conditions (fig. 4), whereas $\alpha V\beta 3$ integrin is present in both situations. These results assign CD47 a new and essential switch function in the receptor complex. One role of CD36 in this process has been eliminated since the examined cells (human umbilical cord vein cells) did not express this receptor molecule. The key role of TSP-1 was shown by blocking apoptosis with TSP-1-neutralizing or receptor-molecule-neutralizing antibodies. TSP-1 activity can thus be assigned solely to the two specific receptor binding RGD- and CBD-sequence motifs [108]. The simultaneous binding of the RGD peptide to the $\alpha V\beta 3$ integrin and the CBD peptide to the CD47 receptor is sufficient for apoptosis induction. This activity is unique to TSP. No other protein has been described containing both peptide

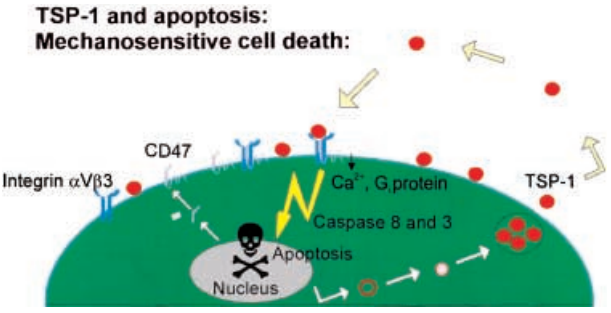


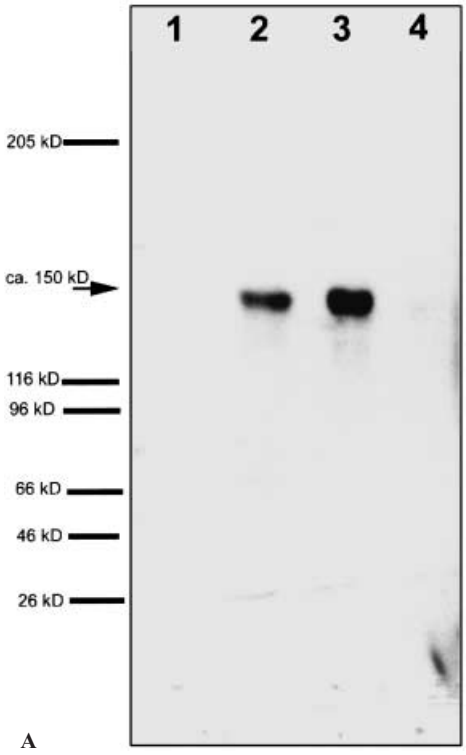
Figure 3. The autocrine loop of TSP-1 and the $\alpha V\beta 3$/CD47 receptor complex. Only on vital endothelial cells can the $\alpha V\beta 3$ integrin be detected. Upon changing the cellular environment to nonphysiological conditions, such as static or irregular hemodynamics, the cells begin to express the CD47 protein on their surfaces (purple) and release TSP-1 (red). The CD47 receptor molecules associate with the $\alpha V\beta 3$ integrin (blue) and form the active receptor complex. The binding of TSP-1 to the $\alpha V\beta 3$ integrin via the RGD sequence motif and the interaction of TSP-1 with the integrin-associated protein (CD47) via the CBD lead to a local Ca^{2+} influx. The receptor function of CD47 and participation of a G_i protein is discussed [56]. The apoptotic pathway continues and leads to activation of caspase-3. The activation of the caspase pathway passes on to the last phase of apoptosis, including cell shrinkage, membrane blebbing, chromatin condensation and nuclear fragmentation.

sequences. The above clearly confirms that TSP-1 is the sole mediator of flow-sensitive apoptosis. Hemodynamic force has been proven to be an essential factor in the survival of vascular endothelium. On the other hand, a model of vascular regression can be established in which the lack of hemodynamic forces is the leading stimulus for removal of vasculature, as happens with the removal of the *vena umilicala* in newborn mammals.

New results indicate, moreover, an important role for TSP-1 in pathophysiological mechanisms. Lack of blood flow is a rare and mostly transient phenomenon. Normally there are laminar flow conditions in the circulation system, but irregular flow conditions are permanently present at arterial bifurcations and sites of abnormal vessel morphology [109]. In vivo arteriosclerotic lesions occur predominantly at sites of flow irregularities, which are thought to be pro-atherogenic. Further investigation shows that flow disturbance and apoptosis are coupled by the described autocrine loop of TSP-1 and the $\alpha V\beta 3$/CD47 receptor complex [110] (fig. 3). We would thus assign a key role to TSP-1 in mechanosensitive apoptosis induction in the initiation of arteriosclerosis. This all appears to suggest that the decision to live or die is coupled to the existing mechanical forces on the mechanosensors of endothelial cells.

Summary and conclusions

TSP-1 contains three different sequence motifs involved in apoptosis: the CSVTCG motif located in the type 1 re-



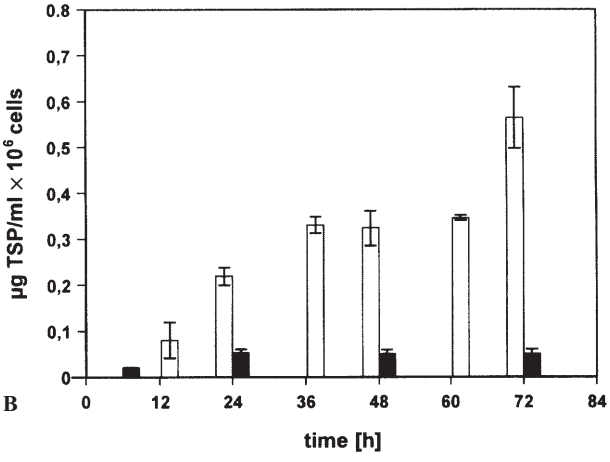


Figure 4. Determination of TSP-1 and the level of secreted TSP-1 under static and dynamic culture conditions. Human umbilical cord vein endothelial cells are prepared and maintained in culture. Cells are seeded in 35-mm cell culture dishes and grown to confluence. (*A*) After the culture medium is changed, the cells are cultivated with serum-free medium for 24 h under static or laminar perfused conditions. Western analysis of TSP-1 expression is done with fresh medium (line 1), static culture medium (line 2), fresh medium containing TSP-1 (line 3) and culture medium from laminar perfused cells (line 4). Proteins are subjected to Western analysis using anti-TSP monoclonal antibody C6.7. (*B*) After the culture medium is changed, the cells are cultivated under static (open bars) or dynamic culture conditions (filled bars) for the next 72 h. At the indicated time, the level of secreted TSP1 is determined by a sandwich enzyme-linked immunosorbent assay (ELISA).

peats, the FYVVMW motif located in the C-terminal cellular binding domain and the RGD motif located in the last type 3 repeat. Each of these motifs addresses a different cellular receptor: CSVTCG binds to CD36, FYVVMW to CD47 and RGD to integrins. The binding of TSP-1 to CD36 seems to be multifunctional, depending on the target cell. It can be an accessory to uptake of apoptotic material by macrophages or apoptosis inducing for microvascular endothelial cells. Although TSP's activity with macrophages depends on the involvement of an integrin thus using its RGD sequence as well, the induction of apoptosis on microvascular endothelial cells has so far been described as a process solely transduced by CD36 without involvement of integrins. A different picture is presented by apoptosis induction via TSP-1 binding to CD47. In the case of endothelial cells lacking CD36, it has clearly been shown that binding to both integrins and CD47 is necessary for induction of apoptosis. At first glance, the studies with B cell leukemia do not seem to fit this conclusion as binding of either TSP-1 or mAbs to CD47 was sufficient to induce apoptosis without integrin signalling. However, soluble TSP-1 as well as soluble mAbs were not active, although both TSP-1 as a trimer and the mAb with its bivalency should have been able to bridge CD47. The proteins were only effective upon immobilization on a surface, suggesting that a multivalent ligand may be required to sufficiently cluster CD47 and activate signalling.

Although the CD36-dependent induction of apoptosis in endothelial cells is restricted to regulation of angiogenesis, the CD47-dependent induction of apoptosis uncovers a new role for TSP-1 in processes regulated by mechanical forces. The mechanosensitive control of TSP-1 expression and action has thus far only been identified in the apoptosis of vascular endothelial cells. Under conditions of regular laminar flow there is no TSP-1 secretion, whereas the withdrawal of this mechanical stimulus results in secretion of TSP-1 at an increasing rate over a period of days. The latter indicates an increased expression of the TSP-1 gene which is supported by the existence of a GAGACC sequence as a potential shear stress-responsive element located at position –1156 in the promotor region of the human TSP-1 gene [111]. The mechanosensitive action of TSP-1 is transduced to the cell through binding to a receptor complex of $\alpha V\beta 3$ integrin and CD47. Even though integrin is expressed constitutionally, the expression of CD47 seems to be regulated by mechanosensitive mechanisms as well. Identification of as yet unknown genomic regulatory DNA sequences should lead to new insights in this area.

It should also prove interesting to see whether the above-described autocrine loop is a general tool of cellular mechano-regulation or whether it is limited to endothelial biology. Fibroblasts have also been shown to be mechanosensitive in that the offset of mechanical tension leads to induction of apoptosis [112]. Initial experiments showed that this induction of apoptosis also occurs via TSP-1 secretion and binding to the integrin/CD47 receptor complex (data not shown). These studies provide further support for the concept that the decision to live or die for many types of cells is coupled with a physiologically identifiable mechanical stimulus recognized by appropriate mechanoreceptors. If a dysfunction in this mechano-regulation with concomitant onset of the apoptotic machinery leads to a pathological situation, as already shown for endothelial cells and proatherogenic flow conditions, then TSP-1 represents a valuable target for pharmaceutical treatment [new findings by Topol et al. 113].

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