
The heparin binding 25 kDa fragment of thrombospondin-1 promotes angiogenesis and modulates gelatinase and TIMP-2 production in endothelial cells

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SPECIFIC AIMS

In this study we addressed the hypothesis that thrombospondin-1 (TSP-1) can exert proangiogenic or anti-angiogenic effects depending on which domain/fragment is functional in a given biological setting. Two fragments of TSP-1 (25 and 140 kDa) generated by the proteolytic cleavage of the intact molecule at a site sensitive to the action of different proteases have been studied *in vivo* in the rabbit cornea assay, and the molecular and cellular mechanism have been investigated *in vitro* on endothelial cells.

PRINCIPAL FINDINGS

1. TSP-1 specifically promotes angiogenesis *in vivo* but neutralizes neovascularization induced by fibroblast growth factor-2 (FGF-2)

Gradient dissemination of TSP-1 into the avascular corneal tissue (200 ng-2 µg/pellet) induced a dose-dependent angiogenic response. The effect was specific since antibodies against either transforming growth factor-β (TGF-β) or FGF-2 did not modify the response, whereas anti-TSP-1 antibodies abolished angiogenesis. Despite its proangiogenic activity, TSP-1 coreleased with FGF-2 inhibited the angiogenic effect of the growth factor, confirming data from other authors.

2. The 25 and 140 kDa TSP-1 fragments have opposite activity on *in vivo* angiogenesis

To identify which domain of TSP-1 was responsible for *in vivo* angiogenesis, the 25 kDa and 140 kDa fragments of TSP-1 were tested in the rabbit cornea

at equimolar concentration with the maximally effective dose of TSP-1 (2 µg/pellet). The heparin binding 25 kDa fragment (285 ng/pellet) elicited a prompt angiogenic response (Fig. 1A) whose extent was even stronger than the one induced by the equimolar concentration of TSP-1. In contrast, the 140 kDa carboxyl-terminal fragment was devoid of any angiogenic activity (Fig. 1B).

When tested in the presence of FGF-2 (200 ng/pellet), the 25 kDa fragment was not inhibitory on FGF-2-induced angiogenesis, but rather increased the number and the growth rate of the newly formed capillaries (Fig. 1C). On the contrary, the 140 kDa fragment blocked the neovascular response induced by FGF-2 (Fig. 1D). These results indicated that the fragmentation of the TSP-1 molecule in smaller proteins can produce a 25 kDa fragment, which accounts for the proangiogenic activity of the whole molecule, and a 140 kDa fragment, which retains the angiostatic effect of TSP-1.

3. TSP-1 and its proangiogenic fragment promote endothelial cell invasion

To clarify the mechanisms underlying TSP-1 and its 25 kDa fragment effect on angiogenesis, both molecules were challenged on endothelial cell invasion across the Matrigel. TSP-1 dose-dependently increased chemoinvasion, whose extent was comparable to that produced by the angiogenic factor FGF-2. In agreement with *in vivo* data, the 25 kDa fragment, but not the 140 kDa, induced endothelial cell inva-

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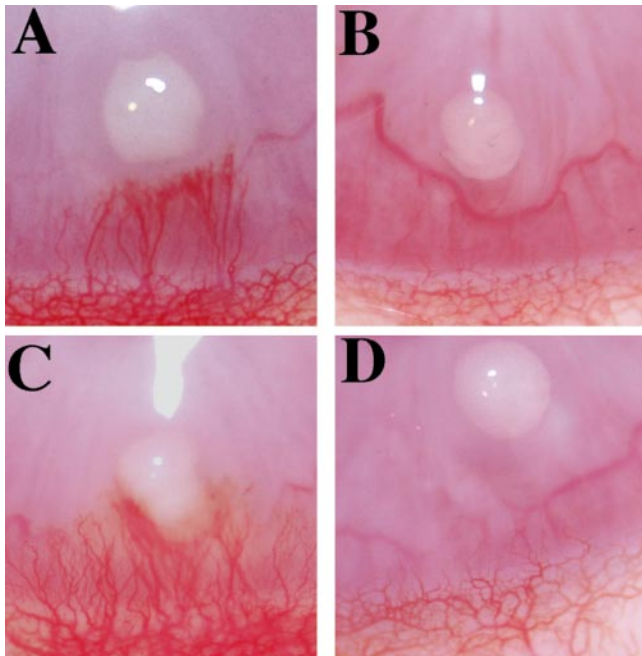


Figure 1. Angiogenic activity of TSP-1 proteolytic fragments. *A, B*) Representative pictures of angiogenesis induced by the 25 (*A*) and the 140 kDa (*B*) fragment of TSP-1 tested at equimolar concentrations with 2 μ g TSP-1. *C, D*) TSP-1 fragments differentially modulate angiogenesis elicited by FGF-2. The 25 kDa (*C*) and 140 kDa (*D*) fragments were tested in the presence of FGF-2 (200 ng). All pictures were taken 12 days after pellet implant. Original magnification 18 \times .

siveness (**Fig. 2**). Heparin, which binds to the 25 kDa fragment with high affinity, strongly prevented TSP-1-induced invasion of endothelial cells, further reinforcing the involvement of the heparin binding domain.

4. MMP-2 and TIMP-2 activity are differentially regulated in endothelium by TSP-1 and its proteolytic fragments

Endothelial cell invasiveness induced by TSP-1 was prevented by inhibitors of matrix metalloproteinase (MMP). Gelatin zymography for MMP production evidenced that TSP-1 stimulated the release of MMP-2 and induced its activation and the appearance of MMP-9 in microvascular endothelial cells. Consistent with the data on chemoinvasion, the 25 kDa fragment increased both latent and activated MMP-2 and induced MMP-9, whereas the 140 kDa fragment did not affect MMP-2 production or activation (inset in Fig. 2).

Since gelatinase activity is the final result of the balance between MMPs and their tissue inhibitors (TIMPs), we assessed whether TSP-1 and its fragments affected the expression of TIMP-2, an inhibitor of MMP-2 activity. Analysis of reverse transcription-polymerase chain reaction (RT-PCR) amplification prod-

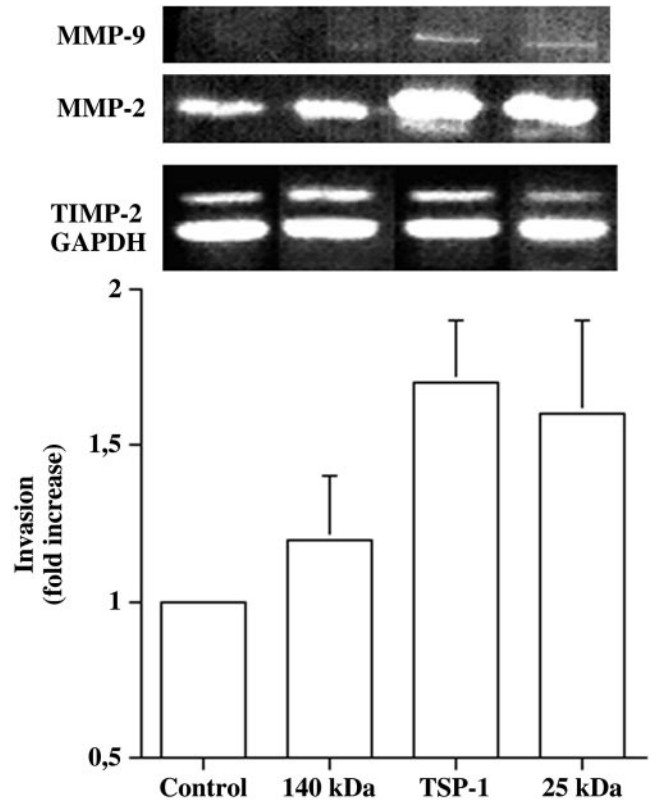


Figure 2. Endothelial cell chemoinvasion in response to TSP-1 fragments. Invasion was assessed in response to equimolar concentration of TSP-1, the 25 kDa and the 140 kDa fragments (110 nM). Data are expressed as the number of migrated cells in 10 high-power fields. *A*) Upper inset: zymographic analysis of conditioned medium from endothelial cells treated with 110 nM TSP-1, the 25 and 140 kDa fragments for 24 h. *B*) Lower inset: RT-PCR analysis of TIMP-2 mRNA expression in endothelial cells exposed to 110 nM TSP-1, the 25 and 140 kDa fragments for 8 h.

ucts of mRNA extracted from cells exposed to TSP-1 or its proteolytic fragments showed that the 140 kDa fragment induced a twofold increase in the expression of TIMP-2, whereas the 25 kDa reduced by 50% its constitutive expression (inset in Fig. 2). This finding indicates that the two fragments affect the proteolytic balance of endothelial cells in opposite ways.

CONCLUSIONS

Angiogenesis is a complex phenomenon resulting from the balance among positive and negative regulators. The present study demonstrates that TSP-1 is a source of smaller mediators of angiogenesis that affect in an opposite way endothelial cell functions and proteolytic activity, thus resulting in a divergent outcome on angiogenesis. We demonstrate that TSP-1 promotes angiogenesis when dismissed in the avascular corneal stroma of albino rabbits, but exerts inhibitory activity when angiogenesis is induced in the same tissue by FGF-2. The observed dual behav-

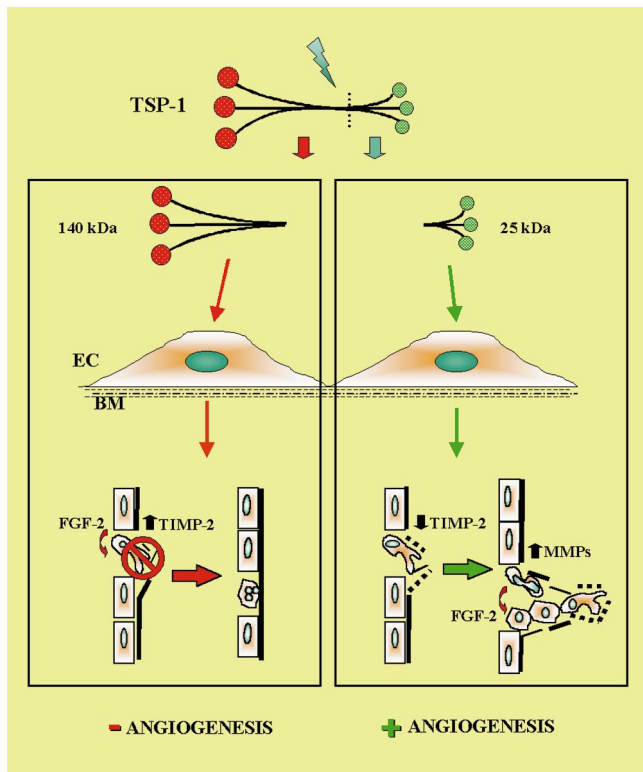


Figure 3. Schematic diagram of the hypothesized TSP-1 involvement in the angiogenic process. After the enzymatic proteolysis of the TSP-1 molecule, the two fragments (25 and 140 kDa) are formed, which exert opposite functions on vascular endothelial cells. Red arrows indicate inhibitory pathways; green arrows indicate stimulatory functions, leading to angiogenesis. EC, endothelial cell; BM, basement membrane.

ior of TSP-1 is produced by domains with opposite functions that also differ in terms of ability to activate degradative and invasive behavior of vascular endothelium. The heparin binding 25 kDa fragment is responsible for the angiogenic activity of the whole molecule, potentiates angiogenesis induced by FGF-2, and increases MMP-2 and MMP-9 production in endothelium while down-regulating TIMP-2 expression. Conversely, the 140 kDa fragment lacks any proangiogenic activity, up-regulates TIMP-2 expression, counteracts TSP-1 proinvasive activity, and inhibits FGF-2-induced angiogenesis (**Fig. 3**). This finding is consistent with other studies that have provided strong evidence that the anti-angiogenic domain of TSP-1 is located within this fragment. On the contrary, our findings provide the first evidence that the other fragment of TSP-1, the 25 kDa heparin binding fragment, is the angiogenic domain of TSP-1. Consistent with the existence of domains with opposite functions within the TSP-1 molecule, we found that the 25 kDa fragment is more efficient

than TSP-1 in promoting angiogenesis *in vivo* and potentiates the FGF-2 effect (**Fig. 3**).

TSP-1 has been reported to stimulate several proangiogenic activities of endothelial cells *in vitro*. It has been shown that TSP-1 induces the production of the matrix metalloproteinase MMP-9 by bovine aortic endothelial cells, thus increasing their ability to invade. In agreement, we found that TSP-1 stimulates the ability of endothelial cells to invade and degrade the extracellular matrix. Moreover, we show that TSP-1 and its fragments regulate the degradative capacity of endothelial cells by acting on MMPs at three levels: transcription, conversion of the proenzyme into the active form, and synthesis of the specific inhibitor TIMP-2.

Despite the evidence provided, the defined molecular/cellular mechanisms for the proangiogenic activity of TSP-1 remain unresolved. Besides a direct activation of endothelial cell functions, the activation of accessory cells cannot be excluded. Moreover, it has been reported that the structural domains of TSP-1 exert the ability to specifically bind to different cell receptors, heparin, matrix components, growth factors and cytokines, proteolytic enzymes, Ca^{2+} . The 25 kDa fragment is characterized by a strong affinity for heparin. Whether the interaction with heparin or heparin-like molecules such as the heparan sulfate proteoglycans (HSPG) is required for the angiogenic activity is not clear. This domain of TSP-1 has been described as interacting with other cell receptors, insensitive to heparinase, and therefore probably is different from HSPG, which also include members of the low density lipoprotein receptor family. We cannot rule out the possible role of other, not yet identified, endothelial cell receptors for the 25 kDa fragment.

In conclusion, our study indicates that the dual role of TSP-1 in angiogenesis might rely on the environmental setting. Depending on which part of the molecule is available and/or functional in a given setting, the resulting effect on angiogenesis will be different. No proof of alternative splicing of TSP has been reported. Nevertheless, fragments of TSP-1 are released *in vivo* by proteolysis of the intact molecule.

Our results contribute to the new concept in angiogenesis that a single molecule acts as a source of both angiogenic and anti-angiogenic peptides. Thus, as described here for TSP-1, the final outcome of a molecule on angiogenesis might depend on its local metabolism, which makes available smaller mediators that affect endothelial cell functions and modulate the MMP/TIMP balance in opposite ways. **[F]**