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INHIBITION OF ANGIOGENESIS BY THROMBOSPONDIN-2

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Summary: To assess the ability of proteins of the thrombospondin family to inhibit angiogenesis, recombinant murine thrombospondin-2, bovine thrombospondin-2/CISP and thrombospondin-5/COMP were purified and tested for ability to block the migration of capillary endothelial cells towards a variety of inducers and to inhibit neovascularization induced in the rat cornea. Both preparations of thrombospondin-2 were active inhibitors in vitro and in vivo whereas thrombospondin-5/COMP was inactive. These results define thrombospondin-2 as a newly identified naturally occurring inhibitor of angiogenesis and suggest that the properdin-like type 1 modules that it shares with antiangiogenic thrombospondin-1 and are missing in thrombospondin-5/COMP could contribute to this activity.

The thrombospondins (TSP) are a family of adhesive molecules secreted by many cells that can influence the attachment, migration and growth of a variety of different cell types. The five family members (1-3) are encoded by separate genes and fall into two distinct classes. TSP-1 and TSP-2/CISP [the bovine form of TSP-2 was originally isolated as Corticotropin-Induced Secreted Protein (4)] are similar homotrimeric molecules. Each subunit contains N- and C-terminal globular domains linked by a central stalk that is composed of four structural modules: a procollagen homology region, three properdin-

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Abbreviations

Thrombospondin family members numbers one through five are abbreviated TSP-1, TSP-2/CISP, TSP-3, TSP-4, and TSP-5/COMP. PARP stands for a proline/arginine-rich protein that defines a module found in several collagens and thrombospondins. Angiogenic factors used in Figure 3 include basic fibroblast growth factor (bFGF), vascular endothelial cell growth factor (VEGF), platelet derived growth factor (PDGF), transforming growth factor beta (TGF-β), interleukin-8 (IL-8) and prostaglandin E1 (PGE1).

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like type 1 repeats, three EGF-like type 2 repeats and seven Ca^{++}-binding type 3 repeats. TSP-3, TSP-4 and TSP-5/COMP [also called Cartilage Oligomeric Matrix Protein (5,6)] form a distinct subgroup of pentameric homopolymers whose subunits lack the procollagen homology region and the type 1 repeats found in TSP-1 and TSP-2. In addition, TSP-5/COMP has a foreshortened aminoterminal domain (2).

TSP-1 has been shown to be a potent inhibitor of the migration, mitogenesis and sprouting of cultured endothelial cells in vitro (7-12) and of neovascularization in vivo (7,8,13). Using proteolytic fragments and synthetic peptides, the angioinhibitory activity of TSP-1 has been mapped to the procollagen homology region (13) and to the type 1 repeats (13,14). Two separate antiangiogenic activities defined by non-overlapping peptides map to the type 1 repeats, one dependent on the ability of the protein to activate TGFβ (14,15) and one independent of TGFβ activation(13).

Examination of the modules present in various TSP family members suggests that TSP-3, TSP-4 and TSP-5/COMP are unlikely to have antiangiogenic activity as they lack the active motifs present in TSP-1. It is less clear what to expect from TSP-2. TSP-2 has a procollagen module containing conserved cysteines, but there is little amino acid homology to TSP-1 within those residues used to derive inhibitory peptides based on TSP-1 (13). In the type 1 module region TSP-2 lacks the sequence motif essential for activation of TGFβ by TSP-1 (15), but does have considerable homology with those residues in TSP-1 from which TGFβ-independent antiangiogenic peptides have been derived (13). To determine if TSP-2 can inhibit angiogenesis, two independent preparations from different species were tested and both showed significant antiangiogenic activity in both in vitro and in vivo assays.

MATERIALS AND METHODS

Proteins: Recombinant mouse TSP-2 was purified from a baculovirus expression system using a protocol suitable for TSP-1 as described (16). TSP-1 was purified in parallel from human platelets (16). Bovine TSP-2/CISP was isolated using a combination of chromatographies from media conditioned by bovine adrenal cortical cells after treatment with ACTH (17). TSP-5/COMP, a kind gift from Paul DiCesare, was purified by him from bovine articular cartilage (6). Both TSP-5/COMP and bovine TSP-2/CISP ran as single bands on an SDS-PAGE gel.

Migration assay: The effect of proteins on the migration of bovine adrenal capillary endothelial cells towards angiogenic stimuli were measured as previously described (13). Briefly, cells were plated on the underside of a gelatinized 5uM nucleopore membrane in a modified Boyden chamber. After adherence, the chamber was inverted, test substances added to the top, and incubation continued for 3-4 hr. The membrane was removed, fixed, stained and the number of cells that had migrated to the top of the chamber counted in 10 high power fields. In a single experiment all samples

were tested in quadruplicate with plating media (Dulbecco's modified Eagles media containing 0.1% bovine serum albumin) serving as a negative control and the same media containing 10 ng/ml bFGF as a positive control. Experiments were repeated two to three times. Toxicity was monitored by treating cells in parallel in a petri dish and measuring trypan blue exclusion at the end of the experiment. For experiments reported here viability was always >95% of untreated controls.

To combine several experiments performed on different days, data was converted to % maximum migration by subtracting the negative control and expressing the data as a percent of migration towards bFGF at 15 μg/ml. Angiogenic factors were purchased from R & D Systems, Minneapolis, MN and used at concentrations determined in preliminary experiments to elicit a maximal response: PDGF at 250 pg/ml, VEGF at 50 pg/ml, TGFβ-1 at 5 to 10 pg/ml, IL-8 at 50 ng/ml. PGE1 from Sigma, St. Louis, MO was used at 100 ng/ml.

Cornea assay: To assess in vivo neovascularization, samples were implanted into the rat cornea (13). Rats were anesthetized with metofane and pellets of about 5 ul consisting of Hydron containing test protein with or without 10 μg/ml of bFGF were implanted into pockets about 1 to 1.5 mm from the limbus. Responses were assessed seven days later as negative if no new vessels grew in towards the pellet, as positive if vessel ingrowth was vigorous and sustained, with most vessels reaching the pellet and as +/- if only few vessels grew into the avascular cornea and rarely reached the pellet.

RESULTS

To compare quantitatively the effects of TSP-2 with those of TSP-1, the capillary endothelial cell migration assay was used. Proteins purified in parallel were tested in the same experiment. Over a wide concentration range, recombinant murine TSP-2 behaved quite similarly to classic human platelet TSP-1 (Figure 1). Both failed to induce endothelial cell migration when tested alone and caused no toxicity as measured by trypan blue exclusion (data not shown) and by the stability of the basal migration rate. Both proteins inhibited migration induced by a gradient of bFGF. Bovine TSP-2/CISP gave similar results (Figure 2). The ED_{50} for TSP-1 was 0.7 μg/ml and was 1.2 μg/ml for recombinant mouse TSP-2 and 0.9 μg/ml for bovine TSP-2/CISP.

TSP-5/COMP was tested for angiogenic activity as an example of those pentameric thrombospondin family members lacking procollagen homology and type 1 repeat domains. At concentrations from 0.01 to 15 μg/ml, it was able to slightly stimulate migration at all concentrations, but the stimulation did not increase with increasing dose. No inhibition of migration towards bFGF or towards TGFβ1 was seen, resulting in an ED_{50} of >15 μg/ml. Extensive dialysis of the protein did not change its activity. Pre-treatment of the endothelial cells for 48 hours with COMP prior to testing did not alter their migratory ability.

In contrast to many other inhibitors of angiogenesis (18), TSP-1 is able to block angiogenesis stimulated by a variety of different inducers. To determine if this trait is

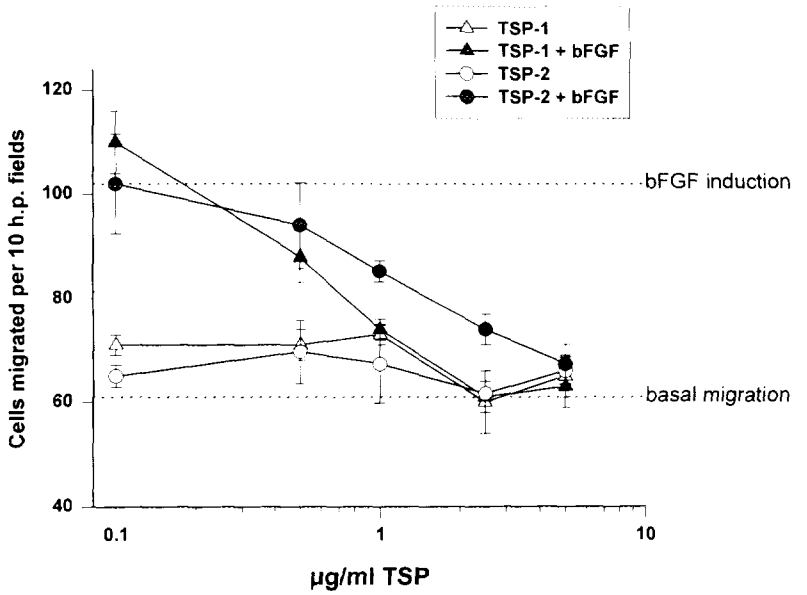

Figure 1. Inhibition of endothelial cell migration by recombinant TSP-2. Recombinant murine TSP-2 and human platelet TSP-1 were assayed at the concentrations indicated either alone for ability to induce migration of capillary endothelial cells or in the presence of a bFGF gradient for ability to inhibit migration induced by this anigogenic factor. Basal migration is the migration seen in the absence of any added compounds. Standard errors are indicated.

shared by TSP-2, a migration assay was performed using a series of inducers that act by a variety of mechanisms to stimulate endothelial cells (Figure 3). All inducers were efficiently inhibited by TSP-1 and most were also inhibited by TSP-2, although TSP-2 was less effective than TSP-1 against VEGF and almost ineffective against TGFβ .

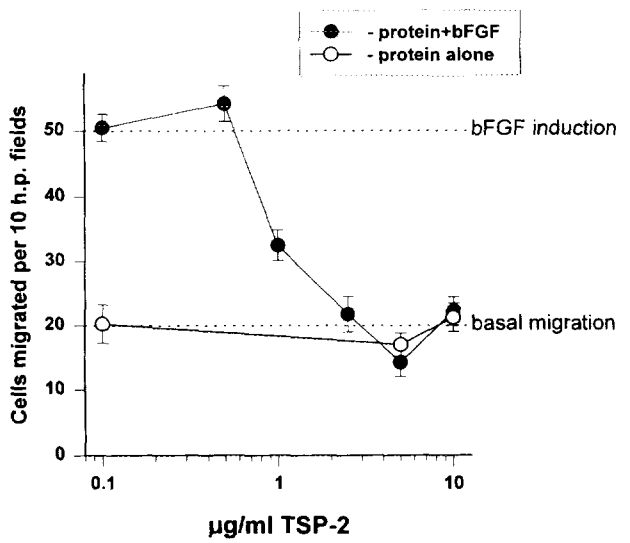

Figure 2. Inhibition of endothelial cell migration by bovine TSP-2/CISP. Bovine TSP-2 was tested as described in legend to Figure 1.

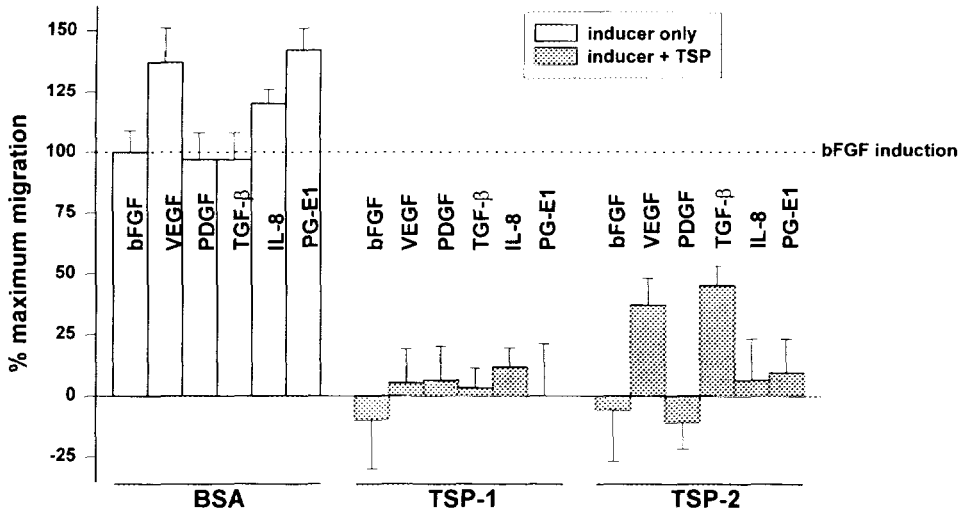

Figure 3. Activity of TSP-1 and TSP-2 against a range of different inducers of angiogenesis. Human platelet TSP-1 at 2.5 μg/ml and bovine TSP-2 at 5 μg/ml were tested for ability to inhibit migration towards a panel of inducers. BSA indicates migrations performed in standard media containing BSA without added TSP.

To determine if the ability of TSP-2 to block endothelial cell migration in vitro reflects truly the ability of TSP family members to block the formation of blood vessels in vivo, the purified proteins were implanted into the rat cornea both alone to assess any independent induction of neovascularization and in the presence of stimulatory bFGF to measure inhibition of angiogenesis (Table 1). All proteins were neutral when tested alone. The single +/- response seen with TSP-5/COMP is most likely due to the pellet having been implanted too close to the limbal vessels. When tested with inducer, TSP-1 inhibited neovascularization at 0.125 μg/cornea, as it has been shown to do in the past (8). Both mouse and bovine TSP-2 showed some inhibition at this dose, but TSP-5/COMP was negative. Complete inhibition of corneal neovascularization by both preparations of TSP-2 could be seen at higher protein concentrations.

DISCUSSION

TSP-2, whether produced recombinantly with baculovirus or purified after its secretion by bovine mammalian cells, inhibited the migration of capillary endothelial cells in vitro and blocked neovascularization in vivo. It was somewhat less potent than TSP-1 when tested in vitro against selected inducers and when tested in vivo where double the amount of protein was required to see complete inhibition of vessel ingrowth. These differences could be the result of differences in stability, for the two molecules do differ in protease susceptibility (16). Alternatively it could be intrinsic to the intact molecule. Of

Table 1. In vivo angiogenic activity of TSP-2 and TSP-5/COMP

Protein	µg/cornea	bFGF	Corneal neovascularization		
			+	+/–	–
TSP-2 (recombinant)	0.125	–	0	0	3
		+	0	2	2
	0.250	–	0	0	2
		+	0	0	3
TSP-2/CISP	0.125	–	0	0	3
		+	0	2	4
	0.320	–	0	0	3
		+	0	0	3
TSP-1	0.125	–	0	0	2
		+	0	0	4
TSP-5/COMP	0.125	–	0	1	2
		+	3	0	0
none		+	6	0	0

the three regions of the TSP-1 monomer that may contribute to its antiangiogenic activity, only one, the TGFβ-independent activity localized to the properdin-like type 1modules, appears from sequence comparisons to be present on the TSP-2 monomer (19). TSP-5/COMP whose subunit lacks all active regions of TSP-1 had no antiangiogenic activity. TSP-5/COMP also lacks a PARP module (20) that the N-terminal globular domains of other thrombospondins share. This module seems unlikely to play a key role, for removing the N-terminal domain containing it from TSP-1 does not reduce its specific activity as an antiangiogenic agent (13).

The ability to act as an inhibitor of angiogenesis is a new function for TSP-2. Although TSP-1 and TSP-2 are very similar in structure and can be secreted simultaneously by the same cells (21,22) and form heterodimers(21), they are differently regulated (22,23) and have distinct patterns of expression in vivo (19,24). The ability of both of them to inhibit angiogenesis is an example of functional redundancy that appears to be based on shared amino acid motifs.

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