Expression and characterization of novel thrombospondin 1 type I repeat fusion proteins

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Thrombospondin (TSP)1 is a trimeric extracellular matrix protein that is held together by two cysteine residues. It is one of five TSP proteins that have been described to date with almost a universal heparin binding capability (TSP5 being the exception). The existence of two conformationally distinct structures in the TSP family (trimers and pentamers) prompted us to investigate the contribution of TSP1 trimeric structure to its inhibitory role in angiogenesis. We expressed full-length recombinant human TSP1, its type I repeats, and murine TSP3 in a human embryonic kidney cell line and evaluated their effect on human dermal microvascular endothelial cell (HMVEC) proliferation and sprouting into tube-like structures *in vitro*. Additionally, two chimaeric molecules were constructed so that the type I repeats

of TSP1 were expressed as either dimers (TSP1–Ig chimaera) or pentamers (TSP1–TSP3 chimaera). Dimeric and pentameric type I constructs are novel structures. We found that, similarly to full-length TSP1, intact trimeric type I repeats were inhibitory to HMVEC angiogenesis *in vitro*. However, dimeric and pentameric type I repeats of TSP1 only partially inhibited HMVEC proliferation and sprouting *in vitro*. TSP3, which is lacking type I repeats, had no inhibitory activity, confirming that type I repeats elicit the anti-angiogenic activity of TSP1.

Key words: angiogenesis, sprouting, trimeric extracellular matrix proteins.

INTRODUCTION

The thrombospondin (TSP) gene family consists of five distinct genes with high degree of homology in their primary structure [1,2]. They are divided into two groups based on their quaternary structure and size. Members of the first group, which comprise TSP1 and TSP2, are homotrimers of 450 kDa each, whereas members of the second group comprise TSP3, TSP4, and TSP5 and are homopentamers [3,4]. The homology between these proteins is manifested by the presence of conserved amino acid sequences known as type I, type II and type III repeats. Both TSP1 and TSP2 have three type I repeats, three type II repeats and seven type III repeats. TSP3, TSP4 and TSP5, on the other hand, are lacking all type I repeats but have an extra type II repeat [2]. Monomeric TSP1 contains two critical cysteine residues at positions 254 and 256, which are believed to mediate trimer formation and assembly [5]. Similarly, two cysteine residues at positions 245 and 248 of monomeric TSP3 stabilize the five-stranded pentamer [4,6]. TSP3 has unique primary and quaternary structures, which suggest a role distinguishing it from that of TSP1. It was found to be developmentally expressed in mouse embryos [2], but no function has been ascribed to TSP3 in adults. TSP1 is an extracellular matrix (ECM) glycoprotein that has been implicated in angiogenesis, the process by which new blood vessels sprout from existing ones. It has been shown to be potentially anti-angiogenic, and was involved in tumour suppression activity as an effector molecule for the tumour suppressor gene p53 [7–9]. The anti-angiogenic activity was localized to the three, 60-residue segments of type I repeats with homology to malarial proteins, properdin and complement proteins C6–C9. Several synthetic peptides derived from this domain have been shown to be potent anti-angiogenic agents

[10,11]. In agreement with these findings, TSP2, the other type-I-repeat-containing protein, was also found to possess anti-angiogenic activity [12]. In a recent study, strong correlation was established between TSP1 level of expression, and tumour angiogenesis and progression in transitional cell carcinoma of the bladder [8]. Nonetheless, several studies reported a pro-angiogenic role for TSP1 in healing wounds and tumour cell lines [13–17]. Moreover, earlier studies on platelet TSP (TSP1) unanimously indicated that TSP promoted cell proliferation, migration and adhesion [18–22], all of which constitute essential components of angiogenesis.

We reasoned that, as far as TSPs are concerned, functional differences among the two groups may be due to at least two other factors (in addition to differences in their primary amino acid sequence): the local level of expression and the oligomeric three-dimensional structure (trimers versus pentamers). The absence of a precise mechanism for TSP1 anti-angiogenic activity and the existence of two highly homologous, yet conformationally distinct protein structures, in the TSP family has prompted us to investigate the relationship between TSP1 type I repeat oligomeric structure and its inhibitory role in angiogenesis.

MATERIALS AND METHODS

Construction and cloning of type-1-repeat chimaeras

The cDNA encoding the N-terminal heparin binding domain (HBD) and the three type I repeats of human TSP1 (bp 180–1829) was obtained by PCR employing the following two oligonucleotides: 5'-CCCTGCTGGGCACCAAAAGCTTCACCA-TGG-3' with a *Hind*III restriction site (underlined), and 5'-

Abbreviations used: TSP, thrombospondin; HMVEC, human microvascular endothelial cell; HBD, heparin binding domain; TIW, recombinant type I repeat; VEGF, vascular endothelial growth factor; ECM, extracellular matrix.

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AAAGCAGGGGACTCGAGAGGCATCAATCAAT-3' with an *XhoI* restriction site and TCA stop codon (bold). This cDNA fragment was directionally subcloned into two mammalian expression vectors pcDNA3 and pCEP4 (Invitrogen, Carlsbad, CA, U.S.A.) utilizing *HindIII* and *XhoI* restriction sites and T4 DNA ligase. This construct was named T1W for type 1 wild.

To create the TSP1-Ig fusion protein, the N-terminal region of human TSP1, consisting of the HBD and type I repeats spanning nucleotides 234–1787 of TSP1, was cloned by PCR using *Taq* DNA polymerase and two synthetic oligonucleotide primers. The upstream primer 5' TCCGTGCTAGCCAACCGCATTC-CAGAGTCT 3' has a *NheI* restriction site (underlined) and is in-frame with the human TSP1 coding sequence. The downstream (antisense) primer 5' ACTGTGCAGATCTTCTGTACATCA-CCAAC 3' has a *BglII* restriction site (underlined) and is in-frame with human TSP1 antisense strand. This cDNA codes for the N-terminal 518 amino acids of TSP1 with a calculated molecular mass of 56 kDa. The CMV-based plasmid, pcDNA3, containing the Fc portion of human IgG fused to the CD5 leader sequence [22a] was digested with *NheI* and *BamHI* and ligated to the *NheI*–*BglII*-digested PCR product. For stable transfections and efficient expression, the TSP1-Ig chimaera was subcloned into the mammalian expression vector pCEP4 using the unique restriction site *XhoI*.

To produce pentameric type I repeats, the N-terminal domain of murine TSP3 consisting of amino acids 1–270 (base pairs 1–810) was obtained by PCR employing a previously cloned mouse TSP3 as template [2], *Taq* DNA polymerase and an upstream primer with an ATG start codon (bold) and a *HindIII* restriction site (underlined): 5'-AGTGAAGCTTCTAAGCGG-CATGGAGAAGCCG-3', and a downstream primer, 5'-AAT-GCTCGTGGCGGCCGCACACCTGACA-3', with a *NotI* restriction site (underlined). The PCR product was directionally cloned in-frame with the human TSP1 cDNA in the plasmid vector pcDNA3 using the restriction sites *HindIII*/*NotI*. This substitution would replace the HBD and the procollagen homology region (bp 1–1206) of human TSP1 (amino acids 1–401) with the pentamer-forming domain of TSP3, but leave intact type I repeats. The identity of the chimaera was confirmed by sequence analysis which showed that the two molecules were in-frame, and by PCR using murine TSP3-specific 5' primer and human TSP1-specific 3' primer. A 180-bp PCR fragment that overlapped the TSP1–TSP3 junction was produced only with template DNA purified from chimaera-transfected HEK-293 cells but not vector-transfected HEK-293 cells. Sequence analysis of the chimaera showed that the last amino acid of the TSP3 peptide was Gly-249 and the first amino acid of TSP1 peptide was Arg-403. All PCR-generated constructs were checked for polymerase infidelity using the automated ALF DNA Sequencer (Pharmacia).

Cell cultures and gene transfer

Cells were plated on to 100-mm plates, at 4.0×10^6 cells/plate, in RPMI or Dulbecco's modified Eagle's media containing 10% (v/v) fetal-bovine serum, 100 units/ml penicillin, 50 µg/ml streptomycin. Cells were transfected with various constructs using TransIT-PanPak kit (Panvera, Madison, WI, U.S.A.) according to the manufacturer's instructions. Pools of resistant clones were expanded after 4 weeks of selection with 800 µg/ml G418 (pcDNA3) and/or 50 µg/ml hygromycin B (pCEP4). Following transfections, cells were incubated in serum-free media supplemented with insulin, transferrin and selenium (Collaborative Research, Bedford, MA, U.S.A.) for 24–72 h and the

media was harvested and treated with a protease inhibitor cocktail consisting of 4-(2-aminoethyl)benzene sulphonyl fluoride, pepstatin A, E-64, bestatin, leupeptin and aprotinin in DMSO (Sigma, St. Louis, MO, U.S.A.). Media was either used without additional manipulation or was subjected to heparin-agarose or Protein A-agarose affinity chromatography to isolate secreted TSP1, TSP3 or their chimaeras.

Expression and purification of the TSP1-Ig chimaera

The TSP1-Ig chimaeric protein was expressed in HEK-293 cells utilizing the plasmid vector pCEP4. Cell-free, serum-free conditioned media from transfected HEK-293 cells were incubated overnight with either heparin-agarose or Protein A/G-agarose on a rotating shaker at 4 °C and then washed extensively with PBS. Heparin-bound proteins were eluted with 20 mM Tris/HCl buffer containing 500 mM NaCl. Protein A/G-bound proteins were eluted as described previously [24]. Briefly, the serum-free media in which transfected HEK-293 cells had been cultured was cleared of cellular debris and mixed with immobilized Protein A/G (Pierce, Rockford, IL, U.S.A.) in a 50-ml conical tube overnight at 4 °C. Protein A/G-agarose beads were washed with ice-cold PBS and bound proteins were eluted with 100 µl of 3.5 M MgCl₂. Samples were separated by analytical SDS/PAGE (10% gel) and gels were stained with Coomassie Blue or subjected to immunoblotting. Eluates containing the purified protein were mixed with 20 ml of PBS, and loaded on to a Centricon Plus-20 (Amicon) unit and centrifuged to attain a final volume of 100 µl. Desalted, concentrated proteins were stored in aliquots at –80 °C.

Expression and purification of TSP proteins

TSP1 and TSP3 cloning into the plasmid vector pCEP4 and transfection into HEK-293 cells was described previously [4]. Stably transfected cells were cultured in serum-containing media for 24 h, washed with serum-free media and incubated in serum-free insulin, transferrin and selenium media for 24–72 h. Media was harvested in 50-ml conical tubes, treated with the protease inhibitor cocktail, and cellular debris was removed by centrifugation. A 50% (w/v) heparin-agarose slurry in PBS (200 µl) was added per 50 ml of media and mixed overnight at 4 °C on a rotating shaker. Agarose beads were washed three times with PBS and bound proteins were eluted with 20 mM Tris/HCl buffer (pH 7.6) containing 500 mM NaCl. Alternatively, conditioned media was concentrated in 20-ml concentrators and the total protein determined.

Human microvascular endothelial cell (HMVEC) sprouting in collagen matrix

HMVEC cells were grown to confluency in CS-5.0 media according to supplier's instructions (Cell Systems, Kirkland, WA, U.S.A.). Cells were treated with trypsin and replated at 1.0×10^5 cells/well in 6-well plates coated with Vitrogen-100 (Collagen Corp., Fremont, CA, U.S.A.) monolayers according to manufacturer's instructions. At the time of plating, cells were left untreated or were treated with TSP1, T1W, TSP1-Ig and TSP1–TSP3 proteins at a final concentration of 100 nM, calculated based on the masses of their respective assumed oligomeric structures. Cells were left to grow on a collagen monolayer for 24 h and were examined for sprouting using light microscopy. The number of sprouting cells (defined as cells with two or more cytoplasmic processes) were counted in 10 random high-power

fields and the means $\pm$ S.D. and the percentage of sprouting cells relative to maximum sprouting was calculated. Maximum sprouting was regarded as the number of HMVEC cells sprouting on the collagen matrix in complete media in the presence of media conditioned with HEK-293 cells transfected with empty vector.

HMVEC proliferation assay

The proliferation assay was as described previously [23]. In brief, cells were treated with trypsin and suspended in complete media containing 10% (v/v) fetal-bovine serum and counted. Approx. 15000 cells were added to each well of a 24-well plate and incubated at 37 °C for 24 h. Fresh media was added to cells and triplicate samples of 500 ng/ml TSP1, TSP3 and type I fusion proteins were added and incubated for 30 min. Finally, 10 ng/ml vascular endothelial growth factor (VEGF) (Sigma) was added to each well and cells were incubated for an additional 72 h. Wells were aspirated, the cells were washed twice with PBS, treated with trypsin and incubated at 37 °C until they ceased to adhere. Viable cells were counted using a haemocytometer and Trypan Blue dye exclusion.

SDS/PAGE and Western blots

Eluted samples were mixed with SDS sample loading buffer, in the presence or absence of 5% (v/v) 2-mercaptoethanol, boiled and separated on either a homogenous 10% polyacrylamide gel or a 3–15% polyacrylamide gradient gel. Following SDS/PAGE, gels were equilibrated with transfer buffer and transferred on to a nitrocellulose membrane using a Multiphor semi-dry electroblotting unit (Pharmacia LKB) at 0.8 mA/cm². Membranes were blocked with 3% non-fat dried milk overnight at 4 °C, washed and probed with domain-specific anti-TSP1 mouse monoclonal antibodies A4.1 (type I-specific), A6.1 (NeoMarkers, Fremont, CA, U.S.A.), and A2.5, or rabbit anti-TSP3 peptide polyclonal antibody RLR (N-terminus recognition epitope RLRGPSRPS), mouse anti-human IgG (Zymed, San Francisco, CA, U.S.A.), and horseradish-peroxidase-conjugated goat anti-mouse or goat anti-rabbit secondary antibodies. Blots were processed using the ECL[®] detection kit (Amersham).

Immunoprecipitation of TSP1–TSP3 chimaera

Transfected HEK-293 cells were lysed for 30 min at 4 °C in 1 ml of lysis buffer (PBS-TDS) consisting of PBS, 1% (v/v) Triton X-100, 0.5% (w/v) deoxycholic acid, 0.1% (w/v) SDS, containing protease inhibitor cocktail. Cell lysates were cleared by centrifugation at 9800 g for 10 min, and soluble proteins were immunoprecipitated with mouse anti-(human TSP1) monoclonal antibody (A4.1) or goat anti-(mouse TSP3) polyclonal antibody (RLR). Protein A/G-agarose slurry (20 μ l) was added to each sample and mixed overnight at 4 °C. The beads were pelleted by centrifugation, washed sequentially with PBS-TDS and PBS, mixed with 40 μ l of sample buffer with or without 2-mercaptoethanol and boiled. Serum-free media conditioned with the same cells was centrifuged to remove cellular debris, treated with protease inhibitor cocktail, mixed with 100 μ l of heparin-agarose slurry and incubated on a rotating shaker overnight at 4 °C. The beads were washed with PBS-TDS twice, with PBS three times and eluted with 90 μ l of 0.2 M Tris/HCl, pH 7.6/500 mM NaCl. Samples were mixed with 10 μ l of 10 $\times$ SDS sample loading buffer, boiled and separated on polyacrylamide gel.

RESULTS AND DISCUSSION

Expression of TSP1 type I repeats

The N-terminal domain of TSP1 consisting of the HBD and type I repeats was expressed and secreted into the media of transfected HEK-293 cells (Figure 1A). In the presence of the reductant, a protein band $\approx$ 56 kDa was recognized in three different clones (C1–C3) using a mouse monoclonal antibody (A2.5) that recognizes the HBD of human TSP1 (Figure 1A, lanes 5, 6 and 7). This antibody failed to recognize any other major protein in the media or in vector-transfected HEK-293 cells (Figure 1A, lanes 1 and 8). In the absence of the reductant, all three protein bands shifted to $\approx$ 160 kDa, suggesting trimeric oligomeric structures (Figure 1A, lanes 2, 3, and 4). Purification of this truncated version of TSP1 utilized its retained heparin-binding capability with ionic strength of 0.35 M, which is comparable with that of full-length TSP1 (0.4 M, results not shown), implying an intact HBD. The correlation between the oligomeric structures of the truncated type I construct and its parent molecule TSP1 was investigated using SDS/PAGE and immunoblotting in the presence or absence of a reductant (Figure 1B). In the presence of 2-mercaptoethanol, T1W and TSP1 consistently migrated as

Figure 1 Expression of type 1 repeats of TSP1

(A) Three separate clones of HEK-293 cells stably transfected with type I construct (C1, C2, C3) and vector-transfected cells (V) were tested for the expression of the T1W protein. Secreted proteins were harvested from the serum-free culture media using heparin-agarose beads, eluted with 0.35 M NaCl in Tris/HCl buffer, separated by SDS/PAGE (8% gel) in the absence (lanes 1–4) or presence (lanes 5–8) of 2-mercaptoethanol and immunoblotted. This Figure is a composite of two separate blots that were probed with either a mixture of A2.5 and A4.1 antibodies (lanes 1–4) or antibody A2.5 (lanes 5–8). (B) T1W, eluted from heparin-agarose beads, and native full-length human TSP1 (Sigma), were separated by SDS/PAGE (6% gel) in the presence (+) or absence (–) of 2-mercaptoethanol and probed with antibody A2.5. Molecular-mass markers are shown on the left.

Figure 2 Expression and characterization of TSP1-Ig chimaera

(A) Western blots of reduced TSP1-Ig chimaera purified using either heparin-agarose and eluted with 0.5 M NaCl (lanes 1 and 5), or Protein A/G-agarose and eluted with 3.5 M MgCl₂ (lanes 2 and 4). Lanes 1 and 2 were probed with mouse monoclonal antibody against human IgG (anti-Fc), lanes 4 and 5 were probed with mouse anti-human TSP1 (A2.5). (B) TSP1-Ig purified on Protein A/G-agarose was also used to characterize the chimaeric molecule under reducing conditions in the presence of 2-mercaptoethanol (+) and under non-reducing conditions in the absence of 2-mercaptoethanol (—). Molecular-mass markers are shown on the left.

single bands of 56 kDa and 180 kDa respectively. However, in the absence of 2-mercaptoethanol TSP1 and T1W migrated as single oligomeric bands of approx. 450 kDa and 200 kDa respectively. Non-reduced T1W most likely assumes a trimeric structure for the following reasons. First, a similar truncated molecule of TSP1, which included the N-terminus 449 amino acid residues (compared with 550 residues of T1W) assumed a trimeric structure [5]. Secondly, TSPs migrate slower in SDS gels than would be expected for their molecular mass [1,2] and, thus, the correlation between monomeric 56 kDa and oligomeric 200 kDa of T1W is well within the 1:3 ratio expected in trimers. Thirdly, the fact that the TSP1 trimeric structure is mediated by the N-terminal domain, which is included in the T1W construct, and is independent of the C-terminus domain [5]. And finally, the observation that T1W binds heparin with comparable affinity to full-length TSP1 (see above).

Expression of the TSP1-Ig chimaera

We also expressed a fusion protein consisting of the HBD, procollagen homology region and the type I repeats of human TSP1 fused to the Fc portion of human IgG. The utilization of the Fc portion of the human IgG molecule as a dimerization domain has been shown to maintain structural integrity but lower biological activity of human deoxyribonuclease I [24]. SDS/PAGE and Western blot analyses of stably transfected HEK-293 cells showed that the TSP1-Ig chimaera was synthesized and secreted into the media, utilizing the CD5 leader sequence (Figure 2A). The identity of the expressed chimaeric protein was confirmed by immunoblotting using mouse antibody against human IgG, which is specific for the Fc portion of the molecule (Figure 2A, lanes 1 and 2), and a mouse antibody specific for the HBD portion of the human TSP1 molecule (Figure 2, lanes 4 and 5). The TSP1-Ig chimaera was purified using both heparin-agarose and Protein A/G-agarose affinity chromatography, utilizing its heparin binding capability and Protein A binding affinity of its Fc domain respectively (Figure 2A). There was a slight difference in the migration pattern of TSP1-Ig chimaera purified using heparin-agarose (Figure 2A,

Figure 3 Expression and characterization of the TSP1-TSP3 chimaera

Immunoblots of purified TSP1-TSP3 fusion protein under oxidized and reduced conditions. (A) The purified protein migrated under reducing conditions as a single band with an apparent molecular mass of 140 kDa (chimer, +) but smeared and co-migrated with human TSP1 under overloaded, non-reducing conditions (chimer, —). (B) However, when separated on an SDS/3–15% gradient polyacrylamide gel, non-reduced TSP1-TSP3 chimaera migrated more slowly than the trimeric human TSP1 (chimer, —), similarly to (C) pentameric murine (m) TSP3 (lane 4) but slower than trimeric murine TSP1 (lane 1) or trimeric human (h) TSP1 (lane 3). Molecular-mass markers (kDa) are shown on the left.

lanes 1 and 5) and Protein A/G-agarose (Figure 2A, lanes 2 and 4). This different migratory pattern was probably due to the high salt content of the Protein A/G-agarose elution buffer in the electrophoresed samples preceding dialysis. The TSP1-Ig chimaera migrated as a 90 kDa band in the presence of the reductant (Figures 2A and 2B), which is consistent with the calculated molecular mass of 84.7 kDa (56.7 kDa type I and 28 kDa Ig). In the absence of a reductant, the chimaeric protein migrated as a 180 kDa band, implying a dimeric conformation (Figure 2B). A second, albeit, less prominent band with an apparent molecular mass of 150–160 kDa, that is probably a partially degraded dimer, was also recognized by the antibody. No higher molecular mass oligomers were observed, although there is a possibility of large oligomeric structures due to disulphide bonding between other cysteine residues [24]. The mature chimaeric protein retained lower heparin binding capability than either T1W or TSP1 and was eluted from heparin-agarose beads with an ionic strength of 0.3 M NaCl (see below).

Expression of TSP1-TSP3 chimaera

A third construct, TSP1-TSP3 chimaera, consisting of the pentamer forming the N-terminal domain of murine TSP3 and human TSP1, lacking the HBD but containing the type I repeats, was synthesized and efficiently secreted into the medium of transfected HEK-293 cells under the TSP3 signal peptide (Figure 3). The mature protein (chimer) migrated with an apparent molecular mass of 140 kDa in the presence of the reductant (Figure 3A, +), in agreement with the expected molecular mass of 144 kDa (based on the amino acid sequence of the monomeric subunit) but much larger than the expected 450 kDa of a trimer in the absence of a reductant (Figure 3B, chimer). Since the N-terminal domain of TSP3 mediates pentamer formation [4,6], it is likely that the TSP1-TSP3 chimaeric molecule assumes a similar pentameric conformation. This is strongly suggested by the fact that the TSP1-TSP3 chimaera, with a comparable monomeric molecular mass to the mouse TSP3 monomer (150 kDa), migrated similarly to pentameric TSP3 (Figure 3C, lanes 4 and 5). This is also supported by the fact that the

Figure 4 Heparin binding of TSP1 chimaeras

(A) Elution profile of recombinant human TSP1–murine TSP3 chimaera (TSP1–TSP3) bound to heparin–agarose beads. (B) Elution profiles of recombinant murine (m) TSP1 and murine TSP3. (C) Elution profile of the TSP1–Ig protein from heparin–agarose beads. The molarity of NaCl used in the elution is shown above the panels. Molecular-mass markers are shown on the left.

TSP1–TSP3 construct retained heparin binding activity comparable with that of TSP3 but not TSP1 (Figure 4).

Heparin binding activities of type I fusion proteins

Type I protein (T1W) retained slightly lower but comparable heparin binding activity with that of the full-length TSP1 protein (results not shown). The significance of this observation is not clear, although it is possible that other lower-affinity heparin binding sequences exist in the missing C-terminus of TSP1. The TSP1–TSP3 chimaeric protein was eluted from a heparin–agarose column with ionic strength similar to that of murine TSP3 (0.3 M) (Figure 4A) but lower than that of murine TSP1 (0.4 M) (Figure 4B). Although TSP1–Ig protein included the HBD of TSP1, its heparin binding capability was less with the lower ionic strength (0.4 M to 0.3 M) (Figure 4C). In all, the three type I constructs retained slightly lower heparin binding capability than the native TSP1 molecule.

Effect of type-I-repeat constructs on HMVEC sprouting

The endothelial cell angiogenic response is partially dependent on the ability of these cells, when induced, to sprout and join end-to-end to form tube-like structures in preparation for new microvessel formation [9,31]. HMVEC cells are indistinguishable from other cells when plated in the presence of attachment factors (A. N. Qabar and J. Bullock, unpublished work). However, HMVECs plated on a collagen matrix develop cytoplasmic processes (sprouts) and link with neighbouring cells (A. N. Qabar and P. J. Polverini, unpublished work). We examined the ability of type I constructs to modify the sprouting of HMVECs by

Figure 5 Inhibition of HMVEC sprouting

HMVECs were cultured on collagen-coated plates in the presence of the indicated constructs. The number of cells with at least two cytoplasmic sprouts were counted in 10 random high-power fields ($\pm$ S.D.) for each treatment. Control is the average number of cells ($n = 10$ fields), cultured in complete media in the absence of any addition, with two or more sprouts, regarded as 100% sprouting.

counting the number of cells that had at least two or more sprouts after plating on collagen matrix-coated plates. The number of cells, in the presence of complete media, was counted in 10 high-power fields and the average was calculated and was taken to be 100% sprouting. Recombinant full-length TSP1 had the most dramatic effect on HMVEC sprouting (Figure 5, TSP1) with more than 75% inhibition of cell sprouting. T1W was as efficient in inhibiting HMVEC sprouting as native full-length TSP1 (Figure 5, T1W), confirming that the source of the anti-angiogenic activity of TSP1 is within this portion of the molecule. However, the TSP1–TSP3 chimaera, which also contains the type I repeats, only partially inhibited sprouting of HMVEC cells (Figure 5, chimer). Thus it seems that a potentially pentameric structure of type I repeats is incapable of conveying equal to or greater than the anti-angiogenic activity of the trimeric oligomer of type I repeats, further suggesting a specific correlation between type I trimeric structure and its anti-angiogenic activity. The affinity-purified TSP1–Ig chimaera only partially inhibited sprouting of HMVEC cells (Figure 5, TSP1–Ig). Neither TSP1–TSP3 nor TSP1–Ig inhibitory activities reached those of recombinant TSP1 (76%) or T1W (72%). Since the procollagen, type II, type III and the C-terminal domains did not exhibit significant anti-angiogenic activity (see above), we concluded that the reduced anti-angiogenic activity of type I construct is not due to the missing C-terminus portion of TSP1, but probably due to the oligomerization of type I repeats into a novel folding with reduced heparin binding capability. In support of this, TSP3, which lacks type I repeats but has a C-terminus homologous to both TSP1 and TSP2, failed to significantly affect HMVEC sprouting (3%; Figure 5). Since the TSP1–TSP3 and TSP1–Ig constructs exerted partial inhibitory activity on HMVEC sprouting, it follows that dimeric and pentameric type I repeats retained some anti-angiogenic activity. The source of such activity is not clear but it may be due to the presence of active surface peptides or basic amino acid residues that interact with ECM proteins independently of any higher oligomeric structure.

Figure 6 Inhibition of HMVEC proliferation

Cells were plated on collagen-coated, 24-well plates in the presence of 10 ng/ml VEGF and approx. 500 ng/ml each of the indicated proteins. After 72 h, the cells from triplicate wells were counted using a haemocytometer and the average ($\pm$ S.D.) was calculated.

Effect of type-I-repeat constructs on HMVEC proliferation

The angiogenic response also involves a surge in endothelial cell proliferation to make up for rapidly migrating cells responding to angiogenic stimuli. To examine the effect of the type-I-repeat constructs on endothelial cell proliferation, the ability of each construct to inhibit HMVEC proliferation in the presence of VEGF was determined (Figure 6). The three type I constructs were capable of partially blocking HMVEC proliferation; however, maximum inhibition was attained with T1W. Trimeric T1W inhibited HMVEC proliferation by 54.8% compared with 16% for TSP1–TSP3 (chimer) and 15.8% for TSP1–Ig. Native TSP1 overexpressed in a PMA-activated human monocytic leukaemia cell line (THP-1), inhibited HMVEC proliferation by 49.4% (Figure 6). However, pentameric recombinant murine TSP3, which lacks type I repeats, was unable to inhibit HMVEC proliferation (5%) (Figure 6, TSP3).

We have expressed the putative, biologically active type I repeats of the ECM protein TSP1 in three different conformations. The fully active native conformation of type I repeats is a trimer consisting of three homologous polypeptide chains. We were able to utilize two defined oligomerization domains to dictate two novel type I conformations in the hope of modifying its inhibitory activity on angiogenesis of HMVECs. The first is the Fc domain of human IgG, which mediates the formation of interchain disulphide bridging and results in the dimeric structure of the IgG molecule. The second is a specific sequence from the N-terminus of the mouse TSP3 molecule that mediates pentamer formation and is also stabilized by two interchain disulphide bridges [4,25]. The three proteins were synthesized and secreted into the media of transfected HEK-293 cells, which lack TSP expression [2]. Our data suggests that these proteins assume the predicted quaternary structures of dimers, trimers and pentamers. Our results, however, suggest lower inhibitory activity of the two novel structures of type I repeats on two important components of HMVEC angiogenic response, namely, sprouting and proliferation. The ability of type I repeats of TSP1 to inhibit proliferation and sprouting of HMVECs in the absence of type II, type III and the C-terminus of TSP1 implies that these domains are not essential for the anti-angiogenic activity of

TSP1, in agreement with previous findings [26]. Our results also suggest that the anti-angiogenic activity of TSP1 probably occurs during the early stages of angiogenesis, where HMVECs proliferate and sprout in preparation for reconstruction of new microvessels [27].

The inhibitory function of type I repeats of TSP1 on angiogenesis is not without its own controversy. Partially fragmented TSP1 with intact heparin binding capability, has lower inhibitory activity on HMVEC sprouting, in spite of the presence of type I repeats (A. N. Qabar, unpublished work). Moreover, synthetic peptides from type I repeats were found to be potent attachment factors for G361 human melanoma cells [28]. It was suggested that TSP1 may assume two opposing functions, one that promotes and another that inhibits angiogenesis [9,17,29]. Some reports have even suggested that the conflicting functions ascribed to TSP are due to two different sources of HMVECs or to different methodologies [30]. However, a recent report showed that TSP1 pro-angiogenic activities are mediated by interaction with the $\alpha 3\beta 1$ integrin receptor [31]. The present study suggests that it is possible that the two opposing effects of TSP1 on angiogenesis can co-exist and are at least partially dependent on its conformation. This dual effect could be conveyed through specific interactions between active sequences within TSP1 (type I repeats, RGD or WSXW) and other ECM molecules [27,32,33] which are likely to take place in the unfolded conformation. In support of this view, the strategic location of a cysteine residue one residue down the RGD sequence, which has the potential for modulating its interaction with other molecules through S–S bonding [34]. It may be recalled that the low oxygen tension in wounds is the first signal for the pro-angiogenic response [35,36], and, under these conditions, the two interchain disulphide bonds at positions 254 and 256, as well as other intrachain S–S bonds, will be reduced and TSP will most likely assume a monomeric conformation resulting in an unfolded, highly interactive molecule, with accompanying profound effects on angiogenesis [37]. Additionally, a reduced ECM environment may catalyse other interactions paramount to the angiogenic response. For example, Stathakis et al. [38] reported a reductase activity in the media of CHO and HT1080 cells that triggered proteolysis of the serine proteinase plasmin, which resulted in the formation of the angiogenesis inhibitor angiostatin. Finally, the transition between pro- and anti-angiogenic phenotypes of TSP1 may also be augmented by the presence or absence of specific cell-surface receptors [39].

The notion that TSP1 anti-angiogenic activity in a cyclical and reversible process such as angiogenesis, is based not solely on its amino acid sequence but on its dynamic oligomeric structure, which primarily depends on the oxidation status of its environment, among other factors, is a worthy concept that requires further exploration.

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