

# THROMBOSPONDIN-1 INHIBITS KAPOSÍ'S SARCOMA (KS) CELL AND HIV-1 Tat-INDUCED ANGIOGENESIS AND IS POORLY EXPRESSED IN KS LESIONS

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## SUMMARY

Kaposi's sarcoma (KS), a neoplasm often associated with iatrogenic and acquired immunosuppression, is characterized by prominent angiogenesis. Angiogenic factors released by both KS and host cells, as well as HHV-8 and HIV viral products, have been implicated in the pathogenesis of this lesion. Angiogenesis is the result of imbalance among angiogenesis promoters and inhibitors, which disrupts homeostasis. The aim of this study was to investigate the expression and mechanism of KS control of thrombospondin-1 (TSP), a physiological inhibitor of angiogenesis. Immunohistochemical analysis of four KS lesions showed only spotty reactivity for TSP in the stroma and in less than 10 per cent of lesional blood vessels. In addition, the typical KS spindle cells were not stained. In agreement with these findings, decreased levels of TSP were measured with an ELISA assay in the supernatants of cultured KS cells, compared with endothelial cells. *In vitro*, TSP inhibited the endothelial cell proliferation and motility induced by KS cell supernatants. TSP also prevented endothelial cell motility induced by Tat, a product of HIV-1 endowed with angiogenic potential and implicated in the pathogenesis of AIDS-KS. *In vivo*, TSP inhibited the angiogenic activity exerted by Tat in the Matrigel sponge model. These results suggest that TSP down-regulation might be permissive for the development of KS-associated angiogenesis. Copyright © 1999 John Wiley & Sons, Ltd.

KEY WORDS—thrombospondin; Kaposi's sarcoma; angiogenesis; AIDS; vascular tumours

## INTRODUCTION

Kaposi's sarcoma (KS) is a proliferative disease which occurs in different forms: the sporadic and Mediterranean, the African endemic, and an iatrogenic form in immunosuppressed transplant recipients. With the AIDS epidemic, a form associated with HIV infection has been rapidly spreading, affecting at least 20 per cent of AIDS patients.

All forms of KS show similar histological features, including the presence of spindle-shaped cells, inflammatory cells, and prominent vascularization. KS lesions are characterized by the presence of a gamma-herpes virus, HHV-8, and a complex network of cytokines, angiogenic and growth factors, extracellular matrix components, and integrins.<sup>2,3</sup> Several angiogenic factors have been implicated in the pathogenesis of KS, including factors released by the KS spindle cells, by inflammatory cells, by HHV-8-infected cells and, in the case of AIDS-associated KS (AIDS-KS), by HIV-infected cells.<sup>2–4</sup> Among the latter, Tat, the transactivating gene product

of the human immunodeficiency virus type 1 (HIV-1), is thought to play a role in the initiation and aggressiveness of AIDS-KS. The HIV-1 Tat protein, an 86–102 amino acid polypeptide, may be released from Tat-expressing cells, entering nearby cells and transactivating endogenous genes and the HIV-LTR.<sup>5</sup> Moreover, the exogenous Tat protein behaves as a heparin-binding angiogenic factor: it binds to and activates the VEGF receptor KDR, consequently stimulating endothelial cell proliferation, activation and migration *in vitro*, and angiogenesis *in vivo*.<sup>4,6–11</sup> This activity is the most likely explanation for the formation of KS-like vascular lesions in the skin of mice transgenic for Tat.<sup>12,13</sup>

Thrombospondin-1 (TSP), a protein secreted by platelets and several cell types including endothelial and inflammatory cells, belongs to a family of five related glycoproteins.<sup>14</sup> TSP is generally considered a potent physiological inhibitor of angiogenesis. However, due to the modular nature of TSP and its resultant ability to interact with different receptors, growth factors, matrix components, and enzymes, the mechanisms and the exact role of TSP in angiogenesis and in tumour progression are complex and have not yet been completely elucidated.<sup>15–17</sup> TSP reduces endothelial cell motility, proliferation, and tube formation *in vitro*, as well as angiogenesis *in vivo*.<sup>18,19</sup> It induces apoptosis in endothelial cells, but not in other cell types.<sup>20</sup> The production of TSP inversely correlates with malignant progression in

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several tumour types,<sup>21</sup> and TSP loss has been associated with the acquisition of the angiogenic phenotype and the formation of vascularized tumours.<sup>22,23</sup>

The present study was designed to characterize the production of TSP by KS cells *in vivo* and *in vitro* and to analyse the effect of TSP on KS-associated and HIV-Tat-induced angiogenesis.

## MATERIALS AND METHODS

### Reagents

TSP was purified from the supernatants of activated human platelets. Polyclonal anti-human TSP antibodies have been previously described;<sup>24</sup> the monoclonal antibody against human TSP (ahTSP-1) was obtained from the ATCC (HB 8432). The monoclonal antibody to CD31 (5F4) was kindly provided by E. Dejana (Milan, Italy). Human recombinant HIV-1 Tat protein was purchased from Intracel (London, U.K.), heparin from Clarisco (Schwarz Pharma, Milan, Italy), and human IFN $\alpha$ -2a (Roferon-A) from Roche (Milan, Italy). Matrigel was extracted from the murine EHS tumour.

### Immunohistochemistry

Fresh biopsies of four cutaneous KS (three classical and one AIDS-associated), of five cutaneous haemangiomas (two juvenile and three cavernous), and of one cavernous haemangioma of the liver were embedded in Optimal Cryopreserving Tissue (OCT) compound (Miles, Elkhart, IN, U.S.A.), snap-frozen in liquid nitrogen, and stored at  $-80^{\circ}\text{C}$ . Cryostat sections were made and fixed in cold acetone for 10 min. After preincubation with pre-immune horse serum to prevent non-specific binding, slides were incubated with ahTSP-1 (1:2 dilution of culture supernatant) or with anti-CD31 (1:10 dilution of culture supernatant) for 30 min. The slides were incubated sequentially with biotin-conjugated horse anti-mouse immunoglobulin antibodies, followed by avidin-biotin-peroxidase complex (PK 4002; Vector Laboratories, Burlingame, CA, U.S.A.). Each incubation step lasted 30 min with 5-min Tris-buffered saline (TBS) washes between each step. Finally, the sections were incubated in 0.03 per cent  $\text{H}_2\text{O}_2$  with 0.06 per cent 3,3'-diaminobenzidine (BDH Chemicals, Poole, U.K.) for 3–5 min, washed, and counter-stained with haematoxylin. A routine control was included in which the primary antibody was omitted.

### Cells

Primary cultures of AIDS-KS (IST-KS IV, IST-KS XI) and classic KS (IST-KS VIII, IST-KS XVI) were isolated, characterized, and cultured as previously described.<sup>4,25</sup> To prepare the serum-free conditioned medium, cells were washed three times and incubated in serum-free medium for 24 h. The supernatant was then collected, centrifuged, and stored in aliquots at  $-80^{\circ}\text{C}$ . The remaining cells were counted to correct for cell number in the ELISA assay.

Freshly isolated human umbilical vein endothelial cells (HUVEC) were grown in M199 with 10 per cent FCS, 10 per cent newborn calf serum, 6 U/ml heparin, and 50  $\mu\text{g}/\text{ml}$  ECGF. Cells were used between the third and fifth passage. Bovine aortic endothelial cells (BAEC), kindly provided by E. Dejana (Milan, Italy), were maintained in DMEM with 10 per cent FCS.

### ELISA analysis of TSP

The presence of TSP in the serum-free supernatants of KS cells was measured with a two-antibody immunoassay. Plates (MaxiSorp Nunc-ImmunoPlates, Nunc, Denmark) were coated with ahTSP-1 (overnight at  $4^{\circ}\text{C}$ ) and then blocked with 3 per cent BSA for 1 h at room temperature. After incubation with the supernatants or TSP standards (3 h at room temperature), polyclonal anti-TSP antibodies were used as a secondary antibody, followed by peroxidase-conjugated anti-rabbit IgG (Sigma, St Louis, MO, U.S.A.) and 1,2-phenylenediamine (Dako, Glostrup, Denmark) as a chromogen. Human platelet TSP (100–2.5 ng) was used as a standard.

### Chemotaxis assay

The chemotactic response of endothelial cells, BAEC, and HUVEC, to supernatants of KS cells or to Tat was carried out in Boyden chambers as previously described.<sup>4,9,18</sup>

### Proliferation assay

HUVEC or BAEC ( $3 \times 10^3$ ) were plated in 96-well microtitre plates in serum-containing medium. After 24 h, KS cell supernatants were added with or without increasing concentrations of TSP and the cells were incubated for an additional 3 days. Cells were then stained with 0.5 per cent crystal violet in 20 per cent methanol and proliferation was evaluated as previously described.<sup>18,24</sup>

### In vivo angiogenesis assay

We utilized the Matrigel sponge model of angiogenesis.<sup>4,26</sup> Tat (20 ng/ml), TSP (0.02 and 0.002 nM), and heparin (21 U/ml, 200  $\mu\text{g}/\text{ml}$ ) were mixed with unpolymerized liquid Matrigel at  $4^{\circ}\text{C}$  to a final volume of 600  $\mu\text{l}$  and then injected subcutaneously into the flank of male C57BL/6 mice, where it rapidly polymerized to a solid gel. Matrigel with buffer alone was used as a negative control. After 4 days, gels were recovered and the haemoglobin content was measured with the Drabkin reagent kit (Sigma, St Louis, MO, U.S.A.) as previously described.<sup>4</sup>

## RESULTS

### Production of TSP by KS

**Immunohistochemical analysis**—Expression of TSP was investigated in tissue sections of four KS lesions

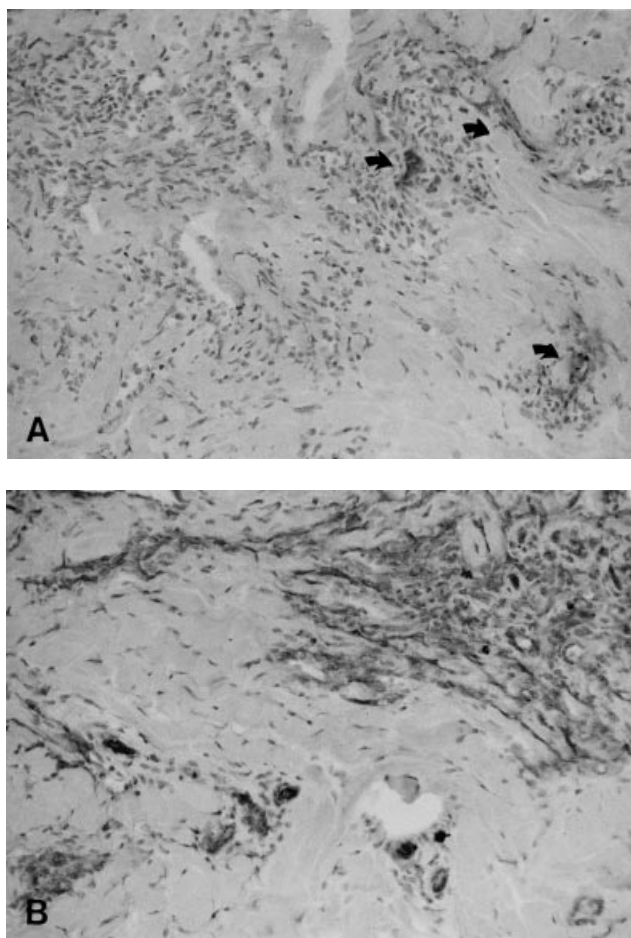


Fig. 1—Cryostat sections of a cutaneous Kaposi's sarcoma immunostained for TSP (ahTSP-1) and CD31 (5F4). (A) Immunostaining for TSP was detected in some KS-associated blood vessels (arrows) and was absent from KS spindle cells ( $\times 200$ , reduced to 77 per cent in printing). (B) CD31 was intensely expressed in the vascular endothelium of all blood vessels and in most KS cells ( $\times 200$ , reduced to 77 per cent in printing; ABC-peroxidase counterstained with haematoxylin)

(two inflammatory and two nodular types). Three patients had classical KS and one was an AIDS-associated form. In all cases, the expression of TSP was confined to spotty areas of the stroma and to some KS-associated blood vessels, comprising less than 10 per cent of total vessels as estimated by CD31 immunostaining (Fig. 1). KS spindle cells were not stained for TSP and were weakly reactive for CD31, as previously reported.<sup>27</sup> A positive control for the reaction was provided by the intense reactivity for TSP in some intralacunar thrombi on the same sections. Immunostaining for TSP was not detected in the stroma or in the vascular endothelium of six benign haemangiomas (not shown).

**ELISA analysis of the supernatants of cultured KS cells**—A double-antibody ELISA assay was used to measure the production of TSP by endothelial cells and cultured KS cells (Table I). Endothelial cells secreted large amounts of TSP ( $1.95 \mu\text{g/ml}$ ). These TSP levels are similar to those described by other authors,<sup>28</sup> confirming the reliability of our assay.

Table I—Production of TSP by cultured KS cells

Cell type	Cell line	TSP ( $\mu\text{g}/10^5$ cells per 24 h)
Kaposi's sarcoma	IST-KS XVI	$0.65 \pm 0.15$
	IST-KS VIII	$0.34 \pm 0.14$
	IST-KS XI	$0.44 \pm 0.17$
	IST-KS IV	$0.85 \pm 0.10$
Endothelium	HUVEC	$1.95 \pm 0.41$

Cells were incubated with serum-free medium for 24 h. TSP levels in the conditioned medium were assessed by ELISA. Results are the mean  $\pm$  SE of at least three independent assays.

The serum-free supernatants of KS cells isolated from four different lesions contained lower levels of TSP than normal endothelial cells (Table I). No difference in TSP production was observed between classic KS (IST-KS VIII, IST-KS XVI) and AIDS-KS (IST-KS XI, IST-KS IV) (Table I).

#### **Effect of TSP on the angiogenic activity of KS supernatants**

KS cell supernatants contain angiogenic factors which stimulate endothelial cell proliferation and motility *in vitro*.<sup>4,25</sup> We investigated whether the addition of TSP to the assays could affect the stimulatory activity of KS supernatants on endothelial cells.

KS supernatants strongly induced the migration and proliferation of BAEC, resulting in an average stimulation of 1.9- and 2.2-fold for motility and proliferation, respectively. TSP (0.2–2 nM) reduced the chemotactic response of BAEC to KS supernatants (up to 73.5 per cent inhibition) (Fig. 2). At higher concentrations, TSP also inhibited BAEC proliferation induced by KS supernatants (Fig. 2).

Similar results were observed with human endothelial cells; KS supernatants increased proliferation by 4.4-fold and TSP (110 nM) inhibited the KS-induced proliferation by 87.8 per cent (not shown).

#### **Effect of TSP on the angiogenic activity of Tat**

The HIV-1 product Tat has been shown to regulate endothelial cell activity and angiogenesis, and is thought to be a regulator of angiogenesis associated with AIDS-KS.<sup>4,6–11</sup> We thus tested whether TSP might affect the angiogenic properties of Tat *in vitro* and *in vivo*. IFN $\alpha$ -activated HUVEC<sup>9</sup> migrated when exposed to Tat protein (Fig. 3). This Tat-dependent migration was fully inhibited by the addition of TSP to the assay (0.02 nM).

The effect of TSP on the *in vivo* angiogenic activity of Tat was evaluated in the Matrigel sponge model, previously used to demonstrate the angiogenic capacity of Tat.<sup>4</sup> The addition of TSP (0.02 and 0.002 nM) to the sponge significantly inhibited the neovascularization induced by Tat (Fig. 4).

## **DISCUSSION**

TSP is considered an inhibitor of angiogenesis, whose production is decreased in several angiogenic tumours.

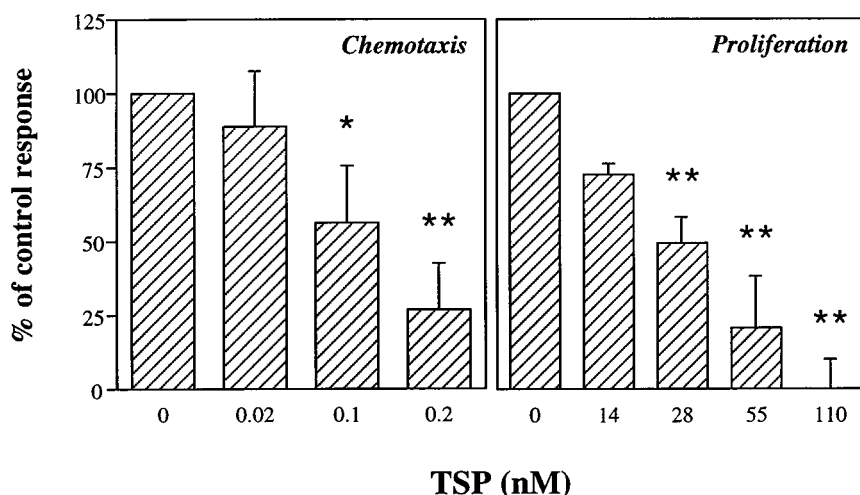


Fig. 2—Effect of TSP on KS-stimulated endothelial cell migration and proliferation. The motility response of BAEC to KS supernatants was assayed in the Boyden chamber; for proliferation, a colorimetric assay was used. TSP was added to the cells at the indicated concentrations. The results, mean and SE of triplicate data from three independent experiments, are expressed as a percentage of the control response. Similar results were obtained with supernatants from different cultures of KS cells. \* $p < 0.05$ ; \*\* $p < 0.001$  compared with controls (Student's  $t$ -test)

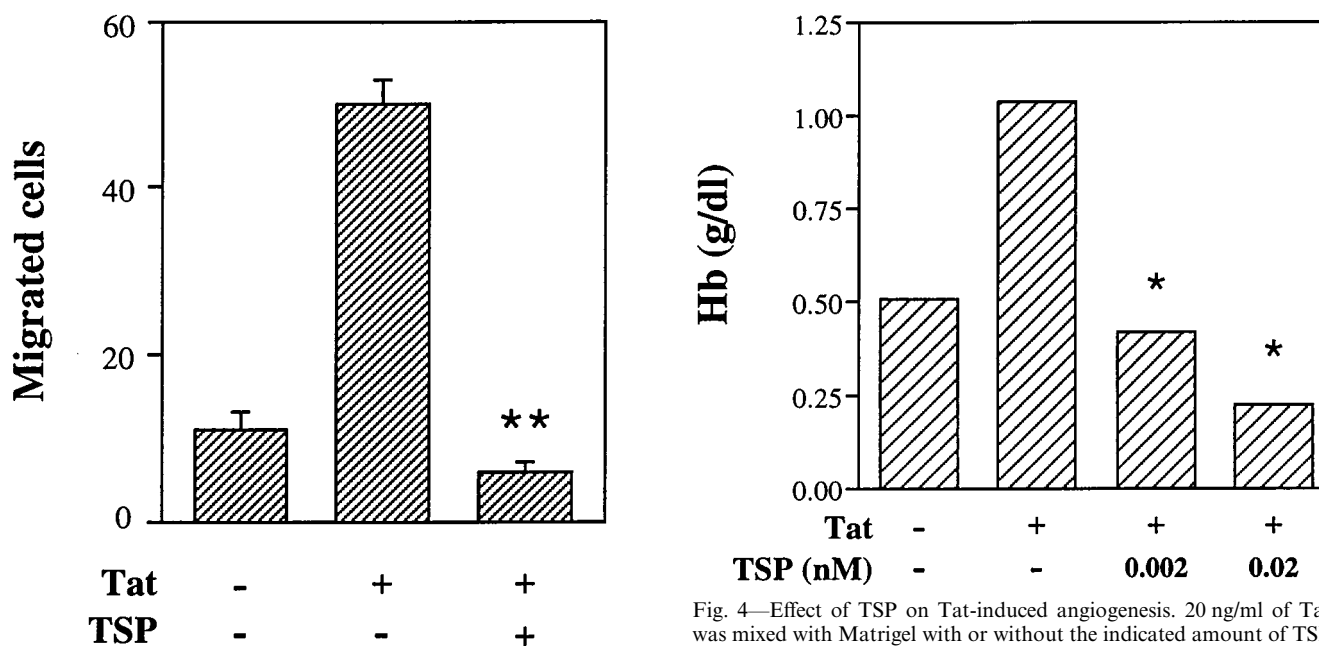


Fig. 3—Effect of TSP on Tat-induced HUVEC migration. The motility response of IFN $\alpha$ -activated HUVEC to Tat (10 ng/ml) was evaluated in the Boyden chamber, in the absence or presence of TSP (0.02 nM). The results are expressed as the number of migrated cells in ten high-power fields, mean and SE of quadruplicates, and are from one experiment representative of three. \*\* $p < 0.0001$  compared with controls (Student's  $t$ -test)

Fig. 4—Effect of TSP on Tat-induced angiogenesis. 20 ng/ml of Tat was mixed with Matrigel with or without the indicated amount of TSP and injected subcutaneously in mice. After 4 days, implants were collected and the haemoglobin content was evaluated as an index of neovascularization. The results are expressed as median values of haemoglobin content of pellets containing no angiogenic stimulus (range of values 0.042–0.103), Tat alone (0.036–2.225), Tat with 0.02 nM TSP (0.067–1.188), or Tat with 0.002 nM TSP (0.107–0.922) ( $n = 8–19$ ). \* $p < 0.05$  compared with controls (Student's  $t$ -test)

However, the correlation between expression of TSP and tumour progression is still debated.<sup>15–17</sup> Several lines of evidence strongly support the hypothesis that the switch to the angiogenic phenotype is accompanied by loss of TSP production. *In vivo* studies on tumour tissues confirmed that tumour cells rarely express appreciable levels of TSP.<sup>29,30</sup> However, stromal cells are strong producers of TSP and the desmoplastic stroma associated with

tumours is often strongly positive, making the overall amount of TSP in these lesions higher than in the normal tissue.<sup>29,30</sup>

KS is an unusual tumour in many respects, including its multifocal origin, the frequent regression found in some KS forms, and the presence of the gamma herpes virus HHV-8 in the lesions.<sup>2,3,31</sup> The latter is able to transform endothelial cells<sup>32</sup> and encodes for several

products involved in cell growth regulation including a G-protein coupled receptor and chemokine analogues with angiogenic properties.<sup>33,34</sup> The histological origin of KS cells, and whether it is a true monoclonal tumour rather than a polyclonal proliferation, is still debated. The maintenance of the lesion requires the continuous presence of a network of cytokines, growth and angiogenic factors, and it is usually benign in non-immunocompromised patients. Angiogenesis results from the imbalance of angiogenesis inducers and inhibitors. This study reports that the angiogenesis inhibitor TSP is poorly expressed by KS cells: (a) no reactivity was observed on immunohistochemical analysis of KS lesions; and (b) lower levels of TSP were detected in the supernatant of cultured KS cells compared with endothelial cells. The lack of TSP production was also observed in six benign haemangiomas, suggesting that down-regulation of TSP expression is associated with endothelial cell proliferation. Interestingly, transformed endothelial cells lines produce little or no detectable TSP<sup>22,35</sup> and restoration of TSP production prevents the formation of vascular tumours in mice.<sup>22</sup>

Several findings suggest that KS spindle cells are derived from the endothelial lineage. KS cells express the endothelial cell markers cadherin-5, CD34, ICAM-1, VCAM-1, and E-selectin, while von Willebrand factor and CD31 are not consistently found.<sup>27,36</sup> A very similar pattern of antigen expression can be observed on endothelial cells exposed to inflammatory cytokines,<sup>36</sup> suggesting that KS spindle cells are similar to cytokine-activated endothelial cells. Interestingly, IL-1 $\beta$  and TNF- $\alpha$  reduce the secretion of TSP by endothelial cells.<sup>37</sup> KS cells thus resemble cytokine-activated endothelial cells in terms also of TSP production.

The pathogenesis and maintenance of KS are strongly dependent on angiogenesis, and inhibitors of angiogenesis are currently under development for the therapy of this lesion. KS cells and HHV-8 Kaposi herpes virus produce factors which stimulate endothelial cell proliferation, motility and invasiveness *in vitro*, and angiogenesis *in vivo*.<sup>4,25,33,34</sup> Our findings show that TSP strongly affects the endothelial proliferation and motility response to KS cell supernatants, suggesting that the lack of TSP production contributes to the strong angiogenic activity of KS cells. The inhibitory activity exerted by TSP occurred at the same concentrations as were previously reported to inhibit endothelial cell proliferation and motility in response to bFGF.<sup>18,19,24</sup>

HIV-1 Tat has been implicated in AIDS-KS.<sup>4,6-13</sup> It has the properties of a heparin-binding growth factor<sup>7</sup> and has been shown to mimic VEGF by binding and activating the KDR receptor.<sup>6</sup> Since TSP interacts with several heparin-binding angiogenic factors, we tested whether TSP would be an effective inhibitor of Tat-induced angiogenesis. TSP strongly inhibited the angiogenic activity of Tat both *in vitro* and *in vivo*. Interestingly, TSP appears to be more potent in inhibiting the angiogenic activity exerted by Tat than that exerted by KS supernatants. The presence of several chemotactic factors in the supernatants of KS cells might explain these differences in efficacy. TSP inhibition of Tat-induced motility of IFN $\alpha$ -stimulated

HUVEC was surprising, since HUVEC, lacking CD36, are not sensitive to the inhibitory activity of TSP.<sup>38</sup> However, preliminary findings suggest that exposure of HUVEC to inflammatory cytokines makes them sensitive to the inhibitory effect of TSP (not shown).

Little is known of the mechanisms of the TSP anti-angiogenic activity. The inhibitory domain of TSP has been located within a 140 kD fragment of TSP.<sup>19,24</sup> We have previously reported that this region of TSP binds to the angiogenic factor bFGF.<sup>24</sup> Since TSP has been reported also to bind HGF/SF, TGF- $\beta$ , proteolytic enzymes, and heparin,<sup>14-17</sup> a direct interaction of TSP with angiogenic factors might represent one mechanism for the inhibition of their activity. Recently, Dawson *et al.* reported that inhibition of endothelial cell migration by TSP occurs through the receptor CD36.<sup>38</sup> In this respect, it is worth noting that KS cells have been reported to express CD36,<sup>39</sup> suggesting that TSP might directly affect KS cell functions. Due to the complex modular nature of this molecule, different mechanisms and receptors might contribute to the anti-angiogenic activity of TSP in both classical and AIDS-KS.

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