Platelet-derived thrombospondin-1 (TSP-1) is a critical negative regulator and potential biomarker of angiogenesis

Running Head: Platelet-derived TSP-1 may be a biomarker

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Abstract

The sequential events leading to tumor progression include a switch to the angiogenic phenotype, dependent on a shift in the balance between positive and negative angiogenic regulators produced by tumor and stromal cells. While the biological properties of many angiogenesis regulatory proteins have been studied in detail, the mechanisms of their transport and delivery in vivo during pathological angiogenesis are not well understood. Here, we demonstrate that expression of one of the most potent angiogenesis inhibitors, thrombospondin-1, is upregulated in the platelets of tumor-bearing mice. We establish that this upregulation is a consequence of both increased levels of thrombospondin-1 mRNA in megakaryocytes, as well as increased numbers of megakaryocytes in the bone marrow of tumor-bearing mice. Through the use of mouse tumor models and bone marrow transplants, we show that platelet-derived thrombospondin-1 is a critical negative regulator during the early stages of tumor angiogenesis. Collectively, our data suggests that the production and delivery of the endogenous angiogenesis inhibitor thrombospondin-1 by platelets may be a critical host response to suppress tumor growth through inhibiting tumor angiogenesis. Further, this work implicates the use of thrombospondin-1 levels in platelets as an indicator of tumor growth and regression.
Introduction

Tumor growth beyond 1-2 mm$^3$ requires the development of new blood vessels, a process known as angiogenesis$^1$. Tumor angiogenesis is triggered by pro-angiogenic regulators, such as vascular endothelial growth factor (VEGF), fibroblast growth factor, and platelet derived growth factor, among others. Host defense against tumor angiogenesis comes in the form of endogenous angiogenesis inhibitors, such as thrombospondin-1 (TSP-1) and endostatin, which counteract the activity of pro-angiogenic factors, and prevent \textit{in situ} carcinomas from undergoing a switch to the angiogenic phenotype$^{2,3}$. The angiogenic response of the host is associated with the increase in the expression of pro-angiogenic proteins that are released from both tumor cells and stromal cells, such as fibroblasts and platelets.

Armand Trousseau made the original connection between platelets and cancer in 1865; however, the role of platelets in tumor angiogenesis remains unclear$^{4,5}$. While platelets circulate throughout the body and participate in the control of hemostasis, increased platelet counts and activation of the coagulation cascade is often seen in cancer patients$^{6,7}$. Additionally, several studies indicate a role for platelets in promoting metastatic disease. These studies utilizing mouse models of metastasis demonstrated that induction of thrombocytopenia, with anti-platelet antibodies or by injecting neuraminidase prior to tumor cell inoculation significantly reduced the formation and overall number of lung metastases$^{8-10}$. Furthermore, animals with dysfunctional platelet receptors, and $NF-E2^{-/-}$ mice, which have virtually no circulating platelets, exhibited a 15-fold reduction in lung metastases, or were completely protected against hematogenous metastasis, respectively$^{11,12}$.

One of the most interesting characteristics of platelets is the large number of biologically active molecules carried in their granules, which can be deposited at sites of vascular injury after
platelet activation. Although platelets are anuclear and lack most of the machinery necessary for protein production, platelets contain a large number of pro- and anti-angiogenic regulators, the majority of which are packaged into alpha granules. By adhering to the endothelium of injured tissues and then secreting their granular contents, platelets deposit high concentrations of angiogenesis regulatory proteins in a regulated and localized manner. Tumor cells are also known to promote platelet activation and aggregation by the release of platelet activating factors, such as thrombin and ADP, resulting in the release of alpha granules and their content, which in turn directly affects tumor growth. Recently, we have described the storage of pro- and anti-angiogenic factors within separate platelet alpha granules while others have demonstrated selective release of either pro- or anti-angiogenic proteins upon platelet activation.

One protein released during platelet activation is TSP-1, a potent anti-angiogenic glycoprotein that constitutes as much as 20% of the total human platelet alpha granule content. In addition to platelets, several cell types express TSP-1, including stromal fibroblasts, endothelial cells, immune cells, and even some tumor cells, and its expression is strongly correlated to wound healing, tissue remodeling and tumor growth. TSP-1 has been shown to inhibit migration, proliferation and induce apoptosis of endothelial cells. TSP-1 also directly affects megakaryopoiesis and the overall vascularity of the bone marrow. Despite remarkable progress over the past 25 years in understanding TSP-1 functions, the diagnostic and therapeutic potential of this molecule is not completely understood.

Recently, the release of platelet TSP-1 has been shown to inhibit thrombopoiesis, diminish bone marrow microvascular reconstruction following myelosuppression, and limit the extent of revascularization in a mouse model of hind limb ischemia, implying that TSP-1 deficient thrombopoietic cells may function as pro-angiogenic agents. If TSP-1 in circulating...
platelets has a direct affect on tumor angiogenesis, the ability to selectively modulate the platelet \(\alpha\)-granule TSP-1 content may assume considerable therapeutic importance. However, the direct effect that platelet TSP-1 has on tumor progression, as well as the mechanism of regulation and delivery of TSP-1 to the site of a growing angiogenic tumor needs to be further investigated.

**Material and Methods**

**Animals**

All animal experiments were performed according to protocols approved by Children’s Hospital Institutional Animal Care and Use Committee. C57Bl/6 animals were purchased from Charles River (North Wilmington, USA). Tsp-1\(^{-/-}\) mice were provided by Dr. Jack Lawler (Beth Israel Deaconess Medical Center, Boston, MA)\(^{25}\). LSL-K-ras\(^{G12D}\) x \(p53^{0/0}\) mice were provided by Dr. Carla Kim (Children’s Hospital, Boston, MA).

**Total body irradiation and bone marrow transplantation**

6 week-old Tsp-1\(^{-/-}\) mice on a C57Bl/6 background were irradiated with a lethal dose of 9.5 Gy from a \(^{137}\)Cs-ray source at a dose of 0.80 Gy/min. Irradiated mice were immediately reconstituted with donor bone marrow cells via tail vein injection. Donor bone marrow cell suspensions were isolated by flushing sterile phosphate-buffered saline into the femurs of 6 week-old wild-type or Tsp-1\(^{-/-}\) mice on a C57Bl/6 background. Single-cell suspensions were prepared by passing the cells through a 30 \(\mu\)m filter, centrifuged and resuspended at a concentration of \(1 \times 10^7\) cells/100 \(\mu\)l. Mice engrafted with saline alone died within one week while successful engraftment was monitored by survival, circulating platelet numbers and expression of TSP-1 in platelets. Platelet TSP-1 expression was assessed by Western blot 6 weeks post-engraftment in Tsp-1\(^{-/-}\) mice and demonstrated comparable levels of platelet TSP-1 expression in
most mice engrafted with wild-type bone marrow (Supplemental Fig. 2) in comparison to wild-type mice.

Migration assay

Cell migration assays were performed using modified Boyden chambers (Transwell-Costar Corp.) coated with 10 mg/ml fibronectin as described previously21. Platelets were collected by retroorbital bleed using heparinized capillary tubes and isolated as previously described5. Resting platelets from wild-type and Tsp-1-/ age matched animals were activated by the addition of 1 unit of thrombin (Sigma-Aldrich), and incubated for 20 min at 37°C, followed by centrifugation for 5 min at 900 RCF at room temperature. Platelet releasate was then transferred into a fresh tube and then added to the endothelial cells used in the migration assay.

Cell Culture

Lewis lung carcinoma and B16F10 mouse melanoma cell lines were obtained from American Type Culture Collection. All cells were maintained in Dulbecco’s modified Eagles’ medium (Cambrex Bioscience) supplemented with 10% fetal calf serum (Cambrex), 100 U/ml penicillin (Cambrex), and 100 µg/ml streptomycin (Cambrex) at 37°C at 5% CO2.

Megakaryocyte isolation and culture

To obtain bone marrow-derived megakaryoblasts, femurs were dissected from Tsp-1-/ mice and the bone marrow was flushed with saline. Single cell suspensions were generated by passage through a 100 µM mesh and then cultured in DMEM supplemented with 10% FBS and 25 ng/ml thrombopoietin (TPO) for 3 days. Cultures were overlaid onto a discontinuous BSA density gradient (0% / 1.5% / 3.0% in PBS) to obtain enriched populations of megakaryocytes.

Platelet isolation
Blood (~900 µl) was collected from anesthetized mice by retroorbital bleeding with heparinized capillary tubes (Fisher scientific) into 0.1 volume of Aester-Jandl anticoagulant (85mM Na Citrate, 69mM citric acid, 20 g/l glucose, pH 4.6) and centrifuged at 1.5 RPM at room temperature for 8 min. The upper phase (~400 µl), platelet rich plasma (PRP), which contains platelets and platelet fragments was transferred into a fresh tube, and platelets were separated by a second centrifugation step at 0.8 RPM for 6 min. Platelets were pelleted by a final centrifugation step at 3.1 RPM for 5 min.

Tumor Growth

6-8 week old mice were inoculated with 1x10^6 tumor cells resuspended in 100 µl of PBS by subcutaneous or intraperitoneal injection. Mice were euthanized 10-14 days after tumor cell inoculation. Tumors were excised, weighed and fixed.

AdenoCre infection

Mice were infected with 5x10^5 units (pfu) of AdenoCre virus at 6 to 8 weeks of age. AdenoCre:CaPi coprecipitates were prepared as described26. Mice were anesthetized with Isoflurane. AdenoCre:CaPi coprecipitates were administered through intramuscular injection.

Quantification of TSP-1 mRNA

Total RNA was isolated from bone marrow megakaryocytes using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol and treated with DNaseI (NEB, Ipswich, MA). RNA samples (1 µg) were reverse transcribed using Invitrogen Superscript III reverse transcriptase according to the manufacturer’s instructions. Amplification was performed in a volume of 25 µl containing 8 µl of template cDNA, 10 µl of real-time DyNAmo HS SYBR Green master mix (Finzymes Inc, Woburn, MA), 5µl of H2O, and 2 µl RT primer set. Amplification was performed for 40 cycles (95°C for 15 seconds, 60°C for 1 minute) on the
The following primers were used: \textit{Tsp-1}, sense: 5'-TCC CCT ATT CTG GAG GGT TC -3', antisense: 5'-TCC CTG GAA ATA GGC ACA AG -3'; \textit{GAPDH}, sense: 5'-ACC ACA GTC CAT GCC ATC AC -3', antisense: 5'-TCC ACC ACC CTG TTG CTG TA -3'. For data analysis the 2$^{-\Delta\Delta C_{T}}$ method\textsuperscript{27} was used with normalization of raw data to the housekeeping gene GAPDH.

**Western Blot Analysis**

Platelets were isolated by retroorbital bleed from wild-type and \textit{Tsp-1}/ mice as previously described and lysed in RIPA buffer (Thermo scientific, Rockford, IL) supplemented with protease inhibitor cocktail (Roche diagnostics LTD, Mannheim, Germany), 10 mM NaF (Sigma, St. Louis, MO), 1 mM Na$_3$VO$_4$ (Sigma) and 1 mM DTT (Sigma). Total protein concentration was quantified and equal amounts of lysates were loaded, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred onto nitrocellulose. Membranes were incubated overnight at 4$^\circ$C with the primary antibodies as follows: TSP-1 (clone Ab-11, Lab Vision, Fremont, CA) diluted at 1:2000, VEGF (clone SC-152, Santa Cruz Biotechnology Inc., Santa Cruz, CA) diluted at 1:500 and β-Actin (Sigma) at 1:5000. Subsequently, membranes were incubated with either goat anti-rabbit or anti-mouse antibodies conjugated to horseradish peroxidase (Amersham Biosciences Corp. Piscataway, NJ) and protein was visualized by enhanced chemiluminescence system (ECL kit; Amersham Biosciences Corp.).

**Immunohistochemistry**

Formalin-fixed, paraffin-embedded tumors or femur sections were deparaffinized by successive incubations in xylene, 100% ethanol, 90% ethanol, 70% ethanol followed by PBS. Epitopes were unmasked with 10 μg/ml proteinase K (Roche diagnostics LTD) in PBS at 37$^\circ$C for 40 minutes and rinsed twice in PBS with 0.3% Triton X-100 (PBS-T). Sections were
immunostained overnight at room temperature by incubation with rat anti-CD31 (BD Pharmingen) diluted at 1:50, or anti-mouse-TSP-1 Ab-4 (Lab Vision) diluted at 1:500 followed by incubation for 1 hour with goat anti-rat or goat anti-mouse Alexa 594-conjugated secondary antibody (1:500; Invitrogen). Nuclei were stained with 1% Hoechst dye for 1 minute.

Isolated platelets and megakaryocytes were fixed with 4% formaldehyde for 20 minutes, attached to poly-lysine coated coverslips and processed for immunofluorescence microscopy. For bone marrow smears, femurs were dissected from mice and bone marrow flushed into a 12 well tissue culture dish.

Bone marrow cells were resuspended in saline and placed in a cytospin with 0.2% gelatin coated slides. Cells were fixed with 4% paraformaldehyde for 10 min at room temperature followed by blocking in 3% milk in TBS-T for 30 min. Anti-TSP-1 mAb (clone Ab-4, Neomarkers) was added for 1 hr at room temperature. Cells were washed with PBS-T before the addition of rabbit anti-mouse-Alexa 594 (1:500; Molecular Probes) for 30 min at room temperature protected from light. Nuclei were stained with 1% Hoechst dye for 1 minute. Cells were extensively washed with PBS-T, mounted, viewed and photographed on a fluorescent microscope.

**Histologic image acquisition and analyses**

Images of bone marrow sections and tumors were taken with a digital camera (AxioCAM HRc, Zeiss) mounted on Zeiss Imager M1 Axio. Plan-Neofluor 10x/0.25, 25x/0.50, and 40x/0.75 objective lenses were used. Images were recorded using Zeiss AxioVision Acquisition software (version 4.5). Images of platelets and megakaryocytes were taken with Nikon TE 2000 Eclipse microscope equipped with a Nikon 100x/1.4 NA objective and a 100-W mercury lamp. Images were acquired with a Hamamatsu (Bridgewater, NJ) Orca IIER CCD camera. Electronic
shutters and image acquisition were under the control of Molecular Devices Metamorph software (Downington, PA). Images were acquired by fluorescence microscopy with an image capture time of 200 to 500 ms.

**Recombinant TSP-1 treatment**

Recombinant human TSP-1 protein was purchased from Protein Sciences Corporation (Meriden, CT). To determine the appropriate concentration of recombinant TSP-1 for our studies, we delivered a range of recombinant TSP-1 (0-10 μg) to Tsp-1−/− mice via tail vein injection. Platelets were isolated by retro-orbital bleed 5 days after TSP-1 treatment and probed for TSP-1 expression. Levels of TSP-1 in platelet lysates correlated with increasing doses of recombinant TSP-1 delivery. 2 μg of recombinant TSP-1 was determined to be the lowest concentration of TSP-1 detected in platelets and therefore this concentration was chosen for further experiments.

Platelets from Tsp-1−/− mice were isolated by retroorbital bleed, resuspended in 1X platelet buffer (10X: 1.45M NaCl, 0.1M Hapes, 5.83mM NaH₂PO₄, 50mM KCl, 20mM MgCl₂, pH 7.4) and incubated with recombinant TSP-1 for 2 hrs at 37°C. Platelets were then extensively washed before being analyzed by either immunohistochemistry, or Western blot analysis.

**RESULTS**

**Thrombospondin-1 expression is increased in circulating platelets of tumor-bearing mice.**

It has been previously hypothesized and more recently demonstrated that circulating platelets act as scavengers and sequester specific angiogenic regulators. To determine whether TSP-1 expression levels in platelets was a direct consequence of a growing tumor, we inoculated both wild type and Tsp-1−/− mice with Lewis lung carcinoma tumor cells that express TSP-1 and
B16F10 melanoma cells that do not express TSP-1. Platelets were collected from tumor-bearing animals when tumors reached 500 mm³. Western blot analysis of platelets isolated from both wild-type and Tsp-1−/− tumor-bearing and non tumor-bearing mice showed increased TSP-1 expression in the presence of tumors that did (Figure 1A, Supplemental Figure 1A) and did not express TSP-1 (Supplemental Figure 1C).

Since we have previously demonstrated that pro- and anti-angiogenic regulators are stored in separate granules in platelets, we examined the expression of TSP-1 in platelets harvested from tumor-bearing and non tumor-bearing wild-type mice by immunofluorescence (Figure 1B). While the platelet VEGF-levels remained unchanged (Figure 1B and Supplemental Figure 1B), the number of TSP-1 reactive granules increased in the presence of a tumor (Figure 1B).

To further confirm the upregulation of TSP-1 expression in platelets during tumorigenesis, we utilized an inducible tumor model. Sarcomas were induced in the LSL-K-rasG12D x p53+/− mice by intramuscular injection of Adenovirus Cre recombinase (Adeno-cre). Platelets were collected every two weeks in mice injected with and without Adeno-cre until tumors reached a volume of 300-500 mm³. Western blot analysis of platelets from tumor-bearing animals demonstrated a significant increase in TSP-1 levels as early as 4.6 weeks post-infection (Figure 1C), further supporting the correlation between platelet TSP-1 levels and tumor size.

**TSP-1 expression in platelets decreases after tumor resection.**

To investigate whether TSP-1 expression in platelets would also reflect tumor regression, we examined TSP-1 expression in platelets after tumor resection (Figure 1D). Wild-type mice were inoculated with Lewis lung carcinoma cells and tumors were resected after reaching ~500 mm³ in volume. Platelets were harvested from mice at various days after tumor resection and platelet
lysates were evaluated for TSP-1 expression. Twenty-one days after tumor resection, the level of TSP-1 in platelets of wild-type mice returned to baseline (Figure 1D). The observed initial upregulation of TSP-1 in the presence of a growing tumor and the subsequent reduction in platelet TSP-1 levels upon tumor resection implicates the potential of platelet TSP-1 as a biomarker of tumor growth and regression.

**Platelet derived TSP-1 delays tumor growth by suppressing angiogenesis.**

To identify the contribution of platelet-derived TSP-1 to tumorigenesis, we performed bone marrow transplants. We transplanted into Tsp-1−/− mice either wild-type or Tsp-1−/− bone marrow and after confirming that platelets from wild-type bone marrow recipients expressed TSP-1 (Supplemental Figure 2A), we inoculated mice subcutaneously with Lewis lung carcinoma cells and monitored tumor growth (Figure 2 and Supplemental Figure 2B). Mice with circulating platelets lacking TSP-1, developed tumors 4-6 days earlier (Figure 2A). Since TSP-1 is a potent angiogenic inhibitor, we quantified tumor angiogenesis in tumors isolated from these mice. These data demonstrated that tumors isolated from mice with Tsp-1 null platelets had significantly higher microvessel density than tumors isolated from mice with wild-type platelets (Figure 2B).

Tumor cells have been shown to promote platelet activation and aggregation by the release of platelet activating factors such as thrombin and ADP. To directly examine the effects of platelet TSP-1 on tumor growth, we inoculated wild-type mice intraperitoneally with luciferase-labeled Lewis lung carcinoma cells that did not express TSP-1. Subsequently, platelets were harvested from either wild-type or Tsp-1−/− mice and were also directly injected into the intraperitoneal cavity with tumor growth monitored by bioluminescence imaging. Previous
studies of endotoxin clearance by platelets demonstrated that intraperitoneal injection of platelets does not result in immediate platelet activation\textsuperscript{32}. Thus, we hypothesized that only direct contact with tumor cells would lead to local deposit of angiogenic regulators from the injected platelets. Since the lifespan of circulating mouse platelets has been shown to be 4.5 days\textsuperscript{33}, we began to monitor tumor growth beginning 72 hrs post-platelet injection and every day afterwards. We observed significant suppression of tumor growth in mice injected with wild-type platelets relative to littermate controls injected with platelet buffer alone at 5 days post-tumor cell inoculation (Figure 2C). Correspondingly, we observed increased tumor volume in the mice injected with $Tsp-1^{-/-}$ platelets as measured by bioluminescence (Figure 2C) and by tumor weight (Supplemental Figure 3A). These data confirm that tumors treated with $Tsp-1^{-/-}$ platelets were significantly larger than those from mice treated with wild-type platelets. To ensure that the inoculated platelets went directly to the tumor site, we also injected GFP-labeled platelets and examined tumors and other organs for GFP expression by immunofluorescence. While we did not detect GFP present on the liver surface, tumors were coated with GFP positive platelets (Supplemental Figure 3B). Using anti-CD31 immunofluorescence to detect endothelial cells, we assessed tumor angiogenesis after treatment with wild-type versus $Tsp-1^{-/-}$ platelets (Figure 2D and E). Tumors from mice injected with wild-type platelets demonstrated significantly decreased microvessel density and high TSP-1 expression. In contrast, tumors from mice injected with Tsp1-null platelets had increased microvessel density and negligible TSP-1 expression (Figure 2D and 2E). These data further support the notion that the loss of TSP-1 converts platelets into pro-angiogenic regulators.
Thrombospondin-1 expression in platelets is critical for modulating its overall angiogenic output.

It has been previously suggested that platelets may be involved in negative regulation of angiogenesis. To examine the angiogenic behavior of platelets and the role of TSP-1, we examined the effect of the entire contents of platelets on endothelial cell migration, a functional assessment of endothelial activation and a pre-requisite of angiogenesis. Platelets from wild-type and Tsp-1/− mice were isolated, activated with thrombin, and the contents were added to endothelial cells. VEGF-induced migration was significantly inhibited in the presence of wild-type platelet contents as compared to the contents from Tsp-1/− platelets (Figure 3A; p < 0.05) and more significantly than treatment with recombinant TSP-1. These data suggest that the loss of TSP-1 expression from platelets abrogated its anti-angiogenic effect.

Since it has been previously demonstrated that platelets are able to scavenge both pro- and anti-angiogenic regulators, we wanted to determine the platelet angiogenic effect in tumor-bearing animals. Once again we performed a migration assay using platelets isolated from tumor-bearing mice and observed a strong inhibitory effect of the platelet releasate on endothelial cell migration (Figure 3B). The increased levels of TSP-1 in platelets from tumor bearing mice further amplified the inhibition of endothelial cell migration.

Megakaryopoiesis and TSP-1 synthesis in megakaryocytes are upregulated during tumor growth.

While our studies suggest that megakaryocytes may directly endocytose TSP-1, it is also possible that the presence of a tumor may stimulate megakaryocytes to synthesize more TSP-1. Almost all the proteins within platelet alpha granules are produced and packaged during platelet
formation and before their release from the megakaryocyte. To investigate the role of megakaryocytes in the production of TSP-1 during tumorigenesis, bone marrow megakaryocytes were isolated from the femurs of mice inoculated subcutaneously with Lewis lung carcinoma cells, after tumors reached 100 and 500 mm³. Western blot analyses revealed significant upregulation of TSP-1 within the megakaryocytes isolated from femurs of tumor-bearing animals (Figure 4A). Quantitative PCR revealed a significant increase in the expression of Tsp-1 mRNA in tumor-bearing mice that correlated with increasing tumor volume (Figure 4B). To determine whether tumor growth affected megakaryocyte production, we isolated bone marrow cells from resting and tumor-bearing wild-type mice and immunostained these cells with anti-GPIBα antibody (a megakaryocyte-specific marker) (Figure 4C). Quantification of GPIBα-positive bone marrow cells demonstrated a significant increase in tumor-bearing mice as compared to non-tumor bearing animals (Figure 4C, p < 0.005). Similarly, Hematoxylin and Eosin staining of femur sections from both wild-type and Tsp-1−/− mice also demonstrated increased megakaryocyte lineage cells in the bone marrow of tumor-bearing animals based on size (Figure 4D). These data indicate that the increase in the total number of circulating platelets (Supplemental Figure 4) may also be due in part, to an increase in megakaryopoiesis observed in tumor-bearing mice.

Platelets acquire Thrombospondin-1 through megakaryocytes.

Anuclear platelets lack the machinery necessary for TSP-1 transcription. Thus, TSP-1 levels can increase in platelets of tumor-bearing mice through several possible mechanisms: direct uptake (endocytosis) by the circulating platelet, uptake (endocytosis) at the megakaryocyte level; and biosynthesis at the megakaryocyte level. To elucidate the mechanism by which platelets
acquired additional TSP-1, and to determine whether recombinant TSP-1 could be endocytosed and sequestered by platelets in vivo, we injected recombinant TSP-1 (rTSP-1) via tail vein into Tsp-1−/− animals and isolated platelets at various times after injection. Western blot analysis of platelet lysates (Figure 5A) indicated that platelets did not directly take up recombinant TSP-1 (rTSP-1) immediately after the injection. However, we did observe the appearance of TSP-1 in circulating platelets 5 days after rTSP-1 injection. Since it has been shown that platelet formation in mice requires 4.7 days before its release from the megakaryocytes33,36, our result suggests that rTSP-1 uptake by platelets may occur via megakaryocyte uptake and packaging into assembling platelets. To rule out the possibility of direct uptake of TSP-1 by platelets, we co-incubated Tsp-1−/− platelets and recombinant TSP-1 ex vivo, followed by anti-TSP-1 immunofluorescence and Western blot analysis (Figure 5B). Our results suggest that platelets are unable to directly endocytose circulating rTSP-1, and therefore require the assistance of megakaryocytes.

We next assessed the role of megakaryocytes in the sequestration of rTSP-1. Bone marrow-derived Tsp-1−/− megakaryocytes were co-incubated with rTSP-1 and analyzed by Western blot demonstrating that megakaryocytes are able to directly endocytose rTSP-1 in vitro (Figure 5C). To examine whether megakaryocytes were able to take up rTSP-1 in vivo, we isolated femurs from Tsp-1−/− mice 24 hours after tail vein injection of rTSP-1. Following megakaryocyte isolation we co-stained cells with anti-GPIBα and anti-TSP-1, and observed TSP-1 expression within the megakaryocytes of mice injected with rTSP-1 versus the saline injected control (Figure 5D). Western blot analysis of megakaryocytes isolated from the bone marrow further confirmed these results, and demonstrated that rTSP-1 was detected within 1 day after rTSP-1 treatment and was still detectable up to 4 days after injection (Figure 5E).
DISCUSSION

The role of endogenous angiogenesis inhibitors in suppressing the progression of in situ tumors and the significance of the balance between the angiogenic output of a tumor versus the host angiogenic defense has been previously described\textsuperscript{2,37}. Since platelets are able to scavenge tumor and stroma-derived angiogenic factors, we wanted to determine if tumor growth within an organism is reflected by the changes in the levels of the angiogenic regulators stored within platelets. We observed an increase in the levels of the potent endogenous angiogenesis inhibitor thrombospondin-1 (TSP-1) in platelets of tumor-bearing mice. This increase in TSP-1 directly correlated with tumor progression, and its expression in platelets resumed to baseline levels within three weeks following tumor resection. The observed inhibition of endothelial cell migration by platelet releasate from the tumor-bearing animals shed some light on the possible functional significance of increase in platelet TSP-1. Since platelets are one of the largest single sources of angiogenic factors in vivo\textsuperscript{38}, we explored the role of platelets and specifically platelet derived TSP-1 in tumor angiogenesis in vivo. Our results suggest that platelet-derived TSP-1 plays a role in the initial stages of tumor growth. Using transplantable tumor models, we demonstrated that the absence of TSP-1 from circulating platelets abrogated the ability of platelets to inhibit tumor angiogenesis in the earliest stages of tumor growth.

The relationship between platelets and malignancies has been studied for over a century, with one of the earliest reports dating back to the 1800’s\textsuperscript{4}. Elevated platelet counts are often associated with poor survival in a variety of cancers, including glioblastomas, gastric, colorectal cancers and gynecological malignancies. There is a strong body of evidence indicating a role for platelets in cancer metastasis, and treatments involving platelet reducing agents have been beneficial in reducing the number of metastatic lesions in several mouse models\textsuperscript{10,11,39,40}. Despite
platelets being one of the largest single sources of angiogenic regulators\textsuperscript{38}, their role in the earliest stages of tumor angiogenesis, regulation and transport throughout the body has not been extensively studied. A single report described the involvement of platelets, and specifically TSP-1, in the regulation of angiogenesis during the reperfusion of ischemic limbs\textsuperscript{24}. This work demonstrated that TSP-deficient megakaryocytes and platelets manifest more robust pro-angiogenic activity than wild-type cells\textsuperscript{24}.

TSP-1 is a potent endogenous angiogenesis inhibitor that normally makes up as much as 10-20\% of the total platelet protein\textsuperscript{16,41}. There have been a limited number of studies investigating TSP-1 regulation, however, down-regulation of TSP-1 has been proposed as a common mechanism by which tumors increase neovascularization\textsuperscript{42}. TSP-1 expression has been shown to be down-regulated by loss of the \textit{p53} tumor suppressor gene\textsuperscript{19} or activation of the \textit{myc} and \textit{ras} oncogenes\textsuperscript{43}. Studies have also shown direct transcriptional upregulation of TSP-1 by \textit{p53}, and transcriptional repression by \textit{c-Myc} and \textit{Id-1}\textsuperscript{44-46}. However, several other factors have been postulated to regulate TSP-1 expression including changes in the expression of pro-angiogenic regulators within the tumor microenvironment, hormone regulation, and TGF-\textbeta, yet no molecular understanding exists of how any of these factors directly or indirectly regulate TSP-1. Under normal physiologic conditions, TSP-1 plasma levels are very low, while in cancer patients and certain mouse models, its levels have been shown to increase\textsuperscript{47-51}. One explanation for the observed increase in the plasma levels of TSP-1 in cancer patients could be its release from tumor-activated platelets, similar to the tumor-induced platelet release of VEGF, another angiogenic factor stored in platelets\textsuperscript{52}. In our study, neither serum nor plasma VEGF or TSP-1 levels in tumor bearing animals correlated with tumor volume (data not shown and Supplemental Fig 5), indicating that the observed TSP-1 plasma increase could be due to non-specific release
Our results suggest that TSP-1 expression is critical for modulating the overall angiogenic function of platelets. We have previously demonstrated that pro- and anti-angiogenic factors are stored within separate platelet granules and that some of these proteins, such as VEGF and endostatin, are selectively released upon platelet activation. Our observation that intraperitoneal injections of platelet contents are sufficient to alter tumor angiogenesis suggests the potent effect of local release of the angiogenic factors from platelets in regulating tumor angiogenesis. The rapid acceleration of tumor growth in wild-type mice bearing Tsp-1 null bone marrow as compared to wild-type bone marrow offers strong evidence that the loss of TSP-1 from platelets shifts the microenvironment to a pro-angiogenic state favoring tumorigenesis. Furthermore, our results suggest that an increase in megakaryopoiesis and the subsequent increase in the numbers of circulating platelets with a concomitant increase in TSP-1 levels in platelets may be one of the earliest host responses to the growth of a tumor. It is fair to speculate that the actual increase in the expression of anti-angiogenic TSP-1 and the platelet ability to deliver it to the site of the growing tumor may be sufficient to alter tumor progression by suppression of tumor angiogenesis. The observed delay in tumor growth of the Tsp-1-/- animals transplanted with wild-type bone marrow as compared to Tsp-1-/- mice transplanted with Tsp-1-/- bone marrow indicates the significance of TSP-1 in the circulating platelets within tumor-bearing animals. Furthermore, the decrease in microvessel density in tumors harvested from mice reconstituted with Tsp-1-/- bone marrow support the conclusion that platelet-derived TSP-1 is critical to suppress tumor angiogenesis.

Blood platelets arise through the formation of proplatelets by the terminally differentiated megakaryocytes within the bone marrow. Since platelets are anuclear, most proteins are either produced within the megakaryocytes or are acquired by platelets while in circulation. Our results
indicate that unlike VEGF (data not shown), platelets are not able to acquire TSP-1 directly from the circulation. The important role of megakaryocytes in packaging TSP-1 into circulating platelets suggests a tight and regulated control over platelet TSP-1. The significance of this observation remains to be further elucidated.

Interestingly, the observed upregulation of TSP-1 and its subsequent increase in circulating platelets in tumor-bearing animals appears to be independent of tumor location. The reduction in platelet levels of TSP-1 following tumor resection suggests a possible future use of platelet TSP-1 expression as a candidate marker for monitoring tumor growth or recurrence. Our results further suggest that tumor-dependent megakaryopoiesis, an increase in the number of circulating platelets and TSP-1 expression may be necessary to suppress tumor angiogenesis. Hence, in the clinical setting, TSP-1 may be a useful universal biomarker of tumor progression and recurrence. The ability to modulate levels of TSP-1 in the platelet α-granules and creation of “designer” platelets may have therapeutic potential in cancer. For instance, one could envision future treatments where in addition to standard anti-angiogenic and chemotherapy, cancer patients could also receive a transfusion of “designer” platelets, where the levels of anti-angiogenic regulators significantly outweigh the levels of pro-angiogenic factors for therapy. Taken together, our data describe a novel mechanism of host suppression of tumorigenesis due to an increase in the number of circulating platelets and an increase in the megakaryocytic expression of TSP-1. Our studies indicate that platelet-derived TSP-1 plays a critical role in suppressing tumor angiogenesis in the earliest stages of tumor growth.
Acknowledgments

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Authorship contributions

A.Z., Primary author of the manuscript, designed and performed experiments, interpreted results and wrote the manuscript. K-H.B. performed experiments, data analysis and interpreted results. R.C.L. performed experiments and interpreted results. S.S. performed experiments, data analysis and interpreted results. J.G. performed experiments. J.F. designed experiments and interpreted results. J.I. Designed experiments, interpreted results, formulated discussions and assisted with manuscript preparation and editing. S.R. Corresponding author, designed experiments, interpreted results, formulated discussions, and wrote the manuscript.

Conflict of interest: No conflict-of-interest to report.

REFERENCES


**Figure Legends.**

**Figure 1.** Thrombospondin-1 (TSP-1) expression in platelets is an indicator of tumor growth and regression. (A) Western blot analysis of TSP-1 expression in equivalent numbers of platelets harvested from wild-type and *Tsp-1*<sup>−/−</sup> mice immediately after inoculation (No tumor) with Lewis lung cells that express TSP-1 and in mice with tumor volumes of 500 mm<sup>3</sup>. β-actin was probed as a loading control. (B) Immunofluorescence analysis of TSP-1 (red) and VEGF (green) expression in platelets harvested from wild-type mice before tumor growth (0) and after tumors reached 500 mm<sup>3</sup> (500). Platelets were immunostained with secondary antibody alone to ensure specificity (data not shown). Bar = 2 μM. (C) Western blot analysis of TSP-1 expression in platelets isolated from an inducible tumor model, *LSL-K-ras<sup>12D</sup> x p53<sup>fl/fl</sup>* mice, after AdenoCre-induced sarcoma formation in the extremities. β-actin was probed as a loading control.
control. Right: Images of $\text{LSL-K-ras}^{G12D} \times p53^{fl/fl}$ mice that were mock infected (uninfected) and 10 weeks post-AdenoCre infection. Red circles indicate sarcomas. (D) Platelet TSP-1 expression by Western blot is probed during tumor growth and after tumor resection on the indicated days. β-actin was probed as a loading control. Right: Quantification of TSP-1 expression relative to β-actin.

**Figure 2. Loss of TSP-1 in platelets leads to accelerated tumor growth and increased tumor angiogenesis.** (A) Lewis lung tumor cells were inoculated in the flank and tumor growth was measured by calipers at the indicated days in lethally irradiated $Tsp-1^{-/-}$ transplanted with either $Tsp-1^{-/-}$ bone marrow (green line) (n=5), or wild-type bone marrow (red line) (n=5). (B) Microvessel density (MVD) per high-powered field (hpf) was quantified in equivalent volume tumors harvested from $Tsp-1^{-/-}$ mice transplanted with wild-type or $Tsp-1^{-/-}$ bone marrow (p<0.005). (C) Bioluminescence images of intraperitoneal tumors in mice following injections of either platelet buffer alone, $1 \times 10^9$ wild-type or $Tsp-1^{-/-}$ platelets. (D) CD31 and TSP-1 immunofluorescence of tumors harvested from mice injected with platelet buffer alone (control), wild-type platelets or $Tsp-1^{-/-}$ platelets. Tumors were immunostained with an antibody against CD31 (left panels) to detect endothelial cells and for TSP-1 (right panels). Bar = 20 μM (E) Microvessel density per high powered field (MVD/hpf) was quantified by CD31 immunostaining in intraperitoneal tumors harvested from mice injected with platelet buffer, wild-type platelets or $Tsp-1^{-/-}$ platelets (p<0.005).

**Figure 3. Endothelial cell migration increases in the presence of $Tsp-1^{-/-}$ platelet lysates.** (A) Endothelial migration was measured in the absence of VEGF (Ctrl) or in response to VEGF
(5 ng/ml) in the presence of thrombin, recombinant TSP-1 (rTSP-1), or platelet lysates from wild-type or Tsp-1<sup>−/−</sup> platelets activated by thrombin (p=0.03). **(B)** Endothelial cell migration was measured in the absence of VEGF (Ctrl.) or in response to VEGF (5 ng/ml) in the presence of thrombin, or thrombin-treated platelet lysates from wild-type mice or tumor bearing (500 mm<sup>3</sup>) wild-type mice. (p = 0.001)

**Figure 4. Megakaryopoiesis and TSP-1 expression is increased in the bone marrow of tumor bearing wild-type mice.** (A) Western blot for TSP-1 expression in megakaryocytes isolated from the bone marrow of Lewis lung tumor bearing mice. β-actin was probed as a loading control. **(B)** Tsp-1<sup>−/−</sup> mRNA levels in bone marrow megakaryocytes were quantified by qPCR and compared to the housekeeping gene GAPDH before tumor growth (0), and when tumors reached 100 mm<sup>3</sup> and 500 mm<sup>3</sup> in volume. **(C)** Immunofluorescence analysis of bone marrow cytospins with anti-GPIBα (red) and Hoechst (blue) to detect megakaryocytes. Bar = 50 μM. Right: Quantification of GPIBα-positive cells in the bone marrow of non-tumor bearing (0) or tumor bearing mice (500 mm<sup>3</sup>). **(D)** Femurs from control and tumor bearing wild-type and Tsp-1<sup>−/−</sup> mice were stained with hematoxylin and eosin. Arrows indicate megakaryocytes. Bar = 50 μM.

**Figure 5. Platelets acquire TSP-1 through megakaryocytes.** (A) Western blot of TSP-1 expression in platelets harvested from Tsp-1<sup>−/−</sup> animals at indicated days after intravenous injection of 2 μg recombinant TSP-1 (rTSP-1). GAPDH was probed as a loading control. **(B).** Left: Platelets from Tsp-1<sup>−/−</sup> mice are immunostained with anti-tubulin (green) and anti-TSP-1 (red) after co-incubation at 37°C for 2 hrs in vitro with 2 μg recombinant TSP-1 (rTSP-1).
Right: Western blot analysis of wild-type platelets, Tsp-1+/ platelets, and Tsp-1+/ platelets after in vitro co-incubation with recombinant TSP-1 (+rTSP-1). GAPDH was probed as a loading control. (C) Western blot analysis of megakaryocytes isolated from the bone marrow of Tsp-1+/ mice after co-incubation at 37°C for 2 hr in vitro with rTSP-1. β-actin was probed as a loading control. (D) Immunostaining with anti-GPIBα (green) and anti-TSP-1 (red) of megakaryocytes isolated from the bone marrow of Tsp-1+/ mice 24 hrs after intravenous injection of saline or 2 μg recombinant TSP-1 (rTSP-1). Bar = 50 μM. (E) Western blot analysis of megakaryocytes isolated from Tsp-1+/ mice 1 and 4 days following intravenous injection of 2 μg recombinant TSP-1. GAPDH was probed as a loading control.
Figure 1

A. wild-type mouse platelets

<table>
<thead>
<tr>
<th></th>
<th>Tsp-1-/- mouse platelets</th>
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<tbody>
<tr>
<td>No tumor</td>
<td>Tumor (500mm³)</td>
</tr>
<tr>
<td>Lewis lung</td>
<td>TSP-1</td>
</tr>
<tr>
<td>(500mm³)</td>
<td>β actin</td>
</tr>
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</table>

B. wild-type mouse platelets

<table>
<thead>
<tr>
<th>Tumor (mm³):</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
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<tr>
<td>500</td>
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IF: anti-VEGF + anti-TSP-1

C. platelets

<table>
<thead>
<tr>
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<th>platelets</th>
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<tbody>
<tr>
<td>0</td>
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</tr>
<tr>
<td>6</td>
<td>8.6</td>
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TSP-1

D. Tumor resected

<table>
<thead>
<tr>
<th>Tumor (mm³)</th>
<th>Days after resection</th>
<th>TSP-1 Relative to β actin</th>
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<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>500</td>
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</table>

TSP-1

2.5

2.0

1.5

1.0

0.5

0.0

Tumor (mm³)

Days after resection

0

500

0

0

0

0

0
Figure 3

A.

![Bar graph showing migration of cells with different treatments (Ctrl, Thrombin, rTSP-1, Wild-type, Tsp-1^−/− platelets) without or with VEGF. The bars represent migrated cells (×10^6) and error bars indicate standard deviation. *p < 0.05.]

B.

![Bar graph showing migration of cells with different treatments (Ctrl, Thrombin, wild-type, wild-type tumor platelets) without or with VEGF. The bars represent migrated cells (×10^6) and error bars indicate standard deviation. **p < 0.005.]

Figure 4

A. Bone marrow megakaryocytes

<table>
<thead>
<tr>
<th>Tumor (mm²)</th>
<th>0</th>
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</thead>
<tbody>
<tr>
<td>TSP-1</td>
<td></td>
<td></td>
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<tr>
<td>β actin</td>
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B. Bone marrow megakaryocytes

<table>
<thead>
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<th>Tumor (mm²)</th>
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<tbody>
<tr>
<td>TSP-1 mRNA expression (relative to GAPDH)</td>
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C. Bone marrow megakaryocytes

<table>
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<tbody>
<tr>
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Number of megakaryocytes

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D. Wild-type bone marrow

<table>
<thead>
<tr>
<th>Tumor (mm²)</th>
<th>0</th>
<th>500</th>
</tr>
</thead>
</table>

| Tsp-1−/− bone marrow |

** p < 0.005
Platelet-derived thrombospondin-1 (TSP-1) is a critical negative regulator and potential biomarker of angiogenesis

Alexander Zaslavsky, Kwan-Hyuck Baek, Ryan C. Lynch, Sarah Short, Jenny Grillo, Judah Folkman, Joseph E. Italiano, Jr. and Sandra Ryeom