Proangiogenic Properties of Thrombospondin-4

Santoshi Muppala, Ella Frolova, Roy Xiao, Irene Krukovets, Suzy Yoon, George Hoppe, Amit Vasanji, Edward Plow, Olga Stenina-Adognravi

Objective—Thrombospondin-4 (TSP-4) is 1 of the 5 members of the thrombospondin protein family. TSP-1 and TSP-2 are potent antiangiogenic proteins. However, angiogenic properties of the 3 other TSPs, which do not contain the domains associated with the antiangiogenic activity of TSP-1 and TSP-2, have not been explored. In our previous studies, we found that TSP-4 is expressed in the vascular matrix of blood vessels of various sizes and is especially abundant in capillaries. We sought to identify the function of TSP-4 in the regulation of angiogenesis.

Approach and Results—The effect of TSP-4 in in vivo angiogenesis models and its effect on angiogenesis-related properties in cultured cells were assessed using Thbs4−/− mice, endothelial cells (EC) derived from these mice, and recombinant TSP-4. Angiogenesis was decreased in Thbs4−/− mice compared with wild-type mice. TSP-4 was detected in the lumen of the growing blood vessels. Mice expressing the P387 TSP-4 variant, which was previously associated with coronary artery disease and found to be more active in its cellular interactions, displayed greater angiogenesis compared with A387 form. Lung EC from Thbs4−/− mice exhibited adhesion, migration, and proliferation capacities compared with EC from wild-type mice. Recombinant TSP-4 promoted proliferation and the migration of EC. Integrin α6 and gabapentin receptor αδ-1 were identified as receptors involved in regulation of EC adhesion, migration, and proliferation by TSP-4.

Conclusion—TSP-4, an extracellular matrix protein previously associated with tissue remodeling, is now demonstrated to possess proangiogenic activity. (Arterioscler Thromb Vasc Biol. 2015;35:00–00. DOI: 10.1161/ATVBAHA.115.305912.)

Key Words: angiogenesis ■ extracellular matrix ■ thrombospondin-4

Our progress in understanding the mechanisms underlying vascular diseases has been heavily focused on the cells involved. Yet it is well established that it is not only the individual cell types but also their interactions with other cells and with the extracellular matrix (ECM) that control the initiation and progression of various vascular pathologies ranging from atherogenesis to angiogenesis.4–6 The ECM is clearly an important regulator of vascular pathologies, but it has only recently become appreciated as a target for pharmacotherapy.

Thrombospondin-4 (TSP-4) belongs to a group of matricellular ECM proteins, which do not provide structural support like collagens or elastins, but instead regulate cell–matrix interactions and functional responses dependent on these interactions, including adhesion, migration, apoptosis, proliferation, and ECM remodeling/fibrosis.6–11 Remodeling of the ECM in the vascular wall initiates and defines the development of cardiovascular disease, diabetic complications, tumor growth, and many other devastating chronic diseases. Remodeling and growth of the blood vessels is guided and regulated by matricellular ECM proteins, which signal through surface receptors to control numerous vascular cell responses. The TSP family consists of 5 proteins (TSP-1 through TSP-5).8 Two members, TSP-1 and TSP-2, are potent antiangiogenic proteins.10–17 However, there have been no reports regarding the angiogenesis-related activities of the other 3 members of the family, with the exception of an observation that TSP-3 does not inhibit angiogenesis.18 Of note, TSP-3, TSP-4, and TSP-5 do not harbor the protein domains that mediate the antiangiogenic activities of TSP-1 and TSP-2.2 Despite recent observations of the presence and critical roles of TSP-4 in the heart, blood vessels, and vascularized tissues,10–18,18 it has not been reported to have any effect on angiogenesis. Circumstantial evidence suggests that TSP-4 might be involved in the regulation of angiogenesis during tissue remodeling and growth. Existing data document the association between TSP-4 and cancer, and tumor growth and metastasis, responses that depend heavily on angiogenesis. Specifically, increased expression of TSP-4 in cancer tissues is associated with cancer progression, and TSP-4 is in the top 1% of the most upregulated genes in several types of cancer, including gastric cancer,25–27 and especially in breast cancer24–30 (www.oncomine.com).

In view of the intimate association between TSP-4, blood vessels, and angiogenic pathologies, the goal of the present work was to examine the effect of TSP-4 on angiogenesis and the proangiogenic functions of endothelial cells (EC).
Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>EC</td>
<td>endothelial cells</td>
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<td>ECM</td>
<td>extracellular matrix</td>
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<tr>
<td>mutTSP4 KI</td>
<td>mutant A387P TSP-4 knock-in</td>
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<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<td>r-TSP-4</td>
<td>recombinant TSP-4</td>
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<td>r-mut-TSP-4</td>
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<td>TSP</td>
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<td>Thbs4−/−</td>
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<td>WT</td>
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Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

Angiogenesis Is Inhibited in Matrigel Plugs in Thbs4−/− Mice

The widely used Matrigel plug model was implemented as an initial approach to assess the effect of TSP-4 deficiency on angiogenesis (Figure 1). Matrigel (750 μl) mixed with 10 ng/ml FGF was injected subcutaneously into wild-type (WT) C57Bl/6 or TSP-4 knockout (Thbs4−/−) mice (n=10). The plugs were excised 7 days later and processed for immunohistochemistry. Sections were stained with anti-CD31 antibodies, and the stained area was quantified. The level of CD31, a marker of EC, was significantly reduced in the plugs excised from Thbs4−/− mice (Figure 1A and 1B). Higher power examination of the sections confirmed that the EC staining was associated with the blood vessels (Figure 1C).

EC were also visualized in Matrigel plug sections using anti-Von Willebrand Factor antibody (Figure 2, green staining), and TSP-4 was visualized using anti-TSP-4 antibody as described previously 9,10,24,31,32 (Figure 1D, red staining). In our previous reports, we described the localization of TSP-4 in blood vessels of different sizes;13 TSP-4 was found in the tunica adventitia of the larger vessels and on the abluminal side of EC in mature capillaries. In contrast to mature vasculature, TSP-4 was found in the lumen of the neovasculature in the Matrigel plugs (Figure 1D).

Angiogenesis Is Inhibited in the Tumor Model in Thbs4−/− Mice

Angiogenesis in WT and Thbs4−/− mice was examined in a tumor angiogenesis model. EMT6, a mouse breast cancer cell line, was propagated in culture, and 1.5×10^6 cells were injected into the mammary fat pad of mice. Tumors were harvested on day 14, weighed, and processed for immunohistochemistry.31 Although we did not detect TSP-4 in cultured EMT6 cells by Western blotting of the cell lysates and cell culture supernatants (not shown), it was detected in the EMT6 tumors in vivo. When tumors from Thbs4−/− mice were stained with anti-TSP-4 antibodies, we have detected TSP-4 staining, suggesting that TSP-4 is produced by EMT6 in vivo tumors (data not shown). There was no difference in tumor weights between WT and Thbs4−/− mice (data not shown). To remove the confounding effect of TSP-4 produced by EMT6 cells in vivo from the model system, we transduced EMT6 cells with lentiviral particles containing TSP-4 shRNA. As a control, EMT6 cells transduced with control shRNA were used. EMT6 cells stably expressing either TSP-4 shRNA or the control shRNA were injected into the mammary fat pads of WT and Thbs4−/− mice, and tumors were collected and processed as described in the Materials and Methods section. In the absence of TSP-4, a significant decrease in tumor weight was detected (Figure 2A). Angiogenesis markers CD31, α-actin, and laminin-1 were visualized by immunostaining with the corresponding antibodies as described in the Materials and Methods section. The levels of all 3 angiogenesis markers were significantly decreased in Thbs4−/− mice (Figure 2B).

A387P Mutation in TSP-4 Promotes Angiogenesis In Vivo

Our previous studies have shown that the P387 variant of TSP-4 interacts with cultured cells with high affinity and induces more extensive signaling than A387 WT TSP-4 form.12,33 A387P has been associated with increased susceptibility to early onset myocardial infarction in several studies.34 The growth of EMT6 tumors grown in mutant A387P TSP-4 knock-in (mutTSP-4) KI mice were significantly larger (Figure 2C) than in WT mice that expressed WT A387 TSP-4. Markers of angiogenesis (CD31, α-actin, and laminin-1) were quantified in tumor sections (Figure 2D). In tumors from mutTSP-4 KI mice, all markers of angiogenesis were significantly increased, consistent with the larger tumor mass and higher proliferating and signaling activity of this variant that we reported previously.12,13

Skin Wound Healing Is Delayed in Thbs4−/− Mice

Wound healing critically depends on angiogenesis and vascular remodeling.35 Changes in angiogenic activity influence the rate of wound healing: suppression of angiogenesis delays and accelerated angiogenesis enhances wound healing.36 We performed skin wound healing assays in WT, Thbs4−/−, and mutTSP-4 KI mice (Figure 3). Full thickness skin wounds (0.7 mm in diameter) were excised on the back of mice and allowed to heal uncovered over time. The sizes of wounds were measured immediately after the surgery and on days 3, 7, 10, and 14 in Thbs4−/− mice (Figure 3A). Wound healing was delayed starting on day 3 after excision in Thbs4−/− mice compared with WT mice. The differences between WT and Thbs4−/− mice were statistically significant on days 3 to 7 when angiogenesis is the most active in the wound.37 At later time points, the wounds still tended to be smaller in the WT mice than in the Thbs4−/− mice, although with healing the size of the wounds became more variable.

We have examined the levels of angiogenesis markers CD31 and α-actin in skin wound sections of WT and Thbs4−/− mice on day 5 (Figure 3B). The difference in the wound size was significantly different between WT and Thbs4−/− mice on day 5 (Figure 3B, left panel). The levels of CD31 and α-actin Activation was significantly lower in Thbs4−/− mice compared with WT mice. The differences between WT and Thbs4−/− mice were statistically significant on days 3 to 7 when angiogenesis is the most active in the wound.37
were both decreased (Figure 3B, middle and right panels), and the difference in the levels of CD31 was statistically significant (Figure 3B, middle panel).

Accelerated Wound Healing in mutTSP-4 KI Mice
The size of wounds was measured on day 5 in WT and mutTSP-4 KI mice (Figure 3C). In mutTSP-4 KI mice, the size of the wounds was significantly different between day 0 and day 5, whereas in WT mice, the difference remained insignificant. The wounds of mutTSP-4 KI mice were significantly smaller than the wounds of WT mice on day 5.

Delayed Postnatal Retinal Vasculature Development in Thbs4−/− Mice
Flat mounts of retinas were prepared and stained with Alexa-568–labeled GS-IB4 as described in the Materials and Methods section to visualize EC. The entire region of vascular outgrowth (region of interest), precise area occupied by
vessels, total vascular length, mean radial vessel extension from the optic nerve, the number of branch points, the whole retinal tissue area (vascular and avascular), and diameter were measured as described in Materials and Methods. These indices characterizing the postnatal development of the retinal vasculature are shown in Figure 4A. The values were normalized to retinal area for each individual flat mount. Retinal area and retinal diameter values were similar between all genotypes. The vascularized area and the mean vessel extension from the center of the optic nerve were significantly smaller in Thbs4−/− mice, whereas the rest of indices also tended to be decreased in these mice (Figure 4A), suggesting that the postnatal development of the retinal vasculature is slower in Thbs4−/− mice.

Accelerated Postnatal Retinal Vasculature Development in mutTSP-4 KI Mice

All of the measured parameters were significantly increased in mutTSP-4 KI mice as compared with both WT and Thbs4−/− mice (Figure 4A), consistent with the observations of increased angiogenesis in these mice in 3 other angiogenesis models. Representative images of retinæ flat mounts from WT, Thbs4−/−, and mutTSP-4 KI mice are shown in Figure 4B.

Regulation of Proangiogenic Activities by TSP-4 in Cultured EC

EC were isolated from the lungs of WT, Thbs4−/−, and mutTSP-4 KI mice, and their angiogenic properties (cell adhesion, cell migration, and proliferation) were compared in vitro.

Cell Migration

The effect of r-TSP-4 and mTSP-4 on EC migration was measured in Boyden chambers. The bottom of the upper chamber was coated with either recombinant TSP-4 (r-TSP-4) or r-mTSP-4 or left uncoated as a control. Both r-TSP-4 and r-mTSP-4 increased the migration of WT EC (Figure 5A). Consistent with the observed effects of r-TSP-4 and recombinant mut-TSP-4 (r-mut-TSP-4), Thbs4−/− EC exhibited decreased migration, and mutTSP-4 KI EC had higher migratory activity compared with WT EC (Figure 5B).

We performed EC migration assays with the function blocking antibodies against known receptors of TSPs as described in Materials and Methods (Figure 5C). All antibodies were well tested blocking antibodies reacting with mouse proteins. As a control, an unrelated antiluciferase antibody, which did not have any effect on EC migration (not shown), was used (Figure 6C, Luc). As was observed in previous experiments (Figure 5A), coating with r-TSP-4 increased migration of EC by 39%±9% (*P=0.0076). We searched for an antibody that produces the specific inhibitory effect of inhibiting the migration on r-TSP-4 without inhibiting the migration on the uncoated surface. Two reagents produced the specific inhibition of TSP-4–induced migration: anti-integrin α2 and gabapentin, a ligand of αδ-1 receptor that prevents TSP-4 binding to this receptor. Both antibodies decreased migration on TSP-4 (P<0.05 as compared with the effect of
a control antibody on migration in the presence of TSP-4), but did not affect the migration in control uncoated wells (no decrease as compared with the effect of a control antibody on migration of EC in control uncoated wells). The difference between the values not normalized to the corresponding controls was also statistically significant for the effects of these 2 antibodies in the presence of TSP-4 and in uncoated wells ($P=0.039$ for $\alpha_2$ integrin antibody; $P=0.0009$ for $\alpha_2\delta_1$ receptor antibody).

Antibodies against integrin $\alpha_V\beta_3$, $\beta_1$ integrin subunit, and CD47 did not have significant effects on EC migration (Figure 5C). Antibodies against $\beta_1$ subunit and $\alpha_\delta_1$ inhibited both the TSP-4–dependent and TSP-4–independent migration ($P<0.05$ when the effect of each of 2 antibodies in the wells coated with TSP-4 was compared with the effect of a control antibody in the wells coated with TSP-4 and when the effect of each of the 2 antibodies on migration in uncoated wells was compared with the effect of a control antibody in the uncoated wells). There was no difference in values not normalized to the corresponding controls: both antibodies reduced migration to the same absolute value both in presence of TSP-4 and in uncoated wells, indicating that the inhibition of migration is not matrix-dependent.

**Cell Proliferation**

The proliferation of EC from WT mice was measured using the CellQuant cell proliferation kit in EC growth medium supplemented with 5% FCS from WT mice with the cells plated onto a fibronectin substrate alone or supplemented with either r-TSP-4 or r-mutTSP-4 (Figure 6A). Both r-TSP-4 and r-mutTSP-4 stimulated EC proliferation. The effect of r-mutTSP-4 was statistically significant as early as 24 hours after the cells were plated (Figure 6A). The effect of r-TSP-4 became significant by 72 hours after EC were seeded.

The proliferation of EC derived from 3 strains of mice (WT, $Thbs4^{-/-}$, and mutTSP-4 KI) was measured at 24, 48, and 72 hours. Proliferation of EC derived from either $Thbs4^{-/-}$ or mutTSP-4 KI mice was slower than those obtained from WT mice (Figure 6B). This pattern was observed with 3 separate isolates of EC from each mouse strain.
Figure 4. TSP-4 regulates the postnatal development of retinal vasculature. The retinae of 5-day-old pups were processed as described in the Materials and Methods section, and the region of vessel area, total vascular length, mean extension of vascularized area form the optic nerve center, and the number of vascular branchpoints were quantified as described in Materials and Methods and normalized to retinal area. A, Analysis of retinal vasculature in flat-mounts stained with Alexa-568 lectin was performed as described in Materials and Methods. Mean±SEM; *Statistically significant difference as compared with WT mice; #Difference significant between TSP4 KO and mutTSP4 KI; n=10 in mutTSP4 KI group; n=16 in WT and TSP4 KO groups. B, Representative images of staining of vessels with Alexa-568 lectin in retinae of 5-day-old mice: Left, WT mice; middle, Thbs4−/− mice; right, mutTSP-4 KI mice. EC indicates endothelial cell; KO, knockout; mutTSP4 KI, mutant A387P TSP-4 knock-in; ROI, region of interest (region of vessel extension from the optical nerve); Thbs4−/−, TSP-4 knockout; and WT, wild-type.
To ensure that proliferation rather than DNA content is changing with genotype, we have counted cells 72 hours after plating the cells in the conditions identical to the conditions described earlier. Equal numbers of cells of all 3 genotypes were plated per culture dish area in the beginning of the experiment. Cells were washed with phosphate buffered saline, fixed with 4% paraformaldehyde, stained with Trypan Blue, and photographed. Stained cell areas were quantified using Adobe Photoshop CS3. The number of pixels of cell staining, which reflects the actual number of cells in the cell culture dish at 72 hours, is presented in Figure I in the online-only Data Supplement. Similar to our data obtained using the CyQuant cell proliferation kit, both WT r-TSP-4 and r-mutTSP-4 increased proliferation (Figure IA in the online-only Data Supplement), and EC from either Thbs4−/− or mutTSP-4 KI mice proliferated more slowly than WT EC (Figure IB in the online-only Data Supplement).

To test whether αβ integrin and αδ-1 contribute to EC proliferation in response to TSP-4, we cultured EC for 72 hours in the presence of either 10 μg/mL neutralizing anti-αβ integrin antibody or 10 μg/mL gabapentin, a ligand of αδ-1 added to the media 6 hours after plating the cells. Antiluciferase antibody was used as a control. Both the anti-αβ integrin antibody and gabapentin, a ligand of αδ-1, significantly decreased proliferation of EC in cell culture plates coated with r-TSP-4 but not in control cell culture plates coated with bovine serum albumin (Figure 6C).
Cell Adhesion

Cell adhesion was measured using the CellQuant cell proliferation kit to quantify the adherent at 1 hour after plating EC in cell culture plates coated with fibronectin mixed with Bovine serum albumin, r-TSP-4, or r-mutTSP-4. r-TSP-4 did not have any effect on EC adhesion (Figure 7A). r-mutTSP-4 was less adhesive for the mouse EC, similar to its effect on human EC as we previously reported.13 When EC from the 3 mouse strains were assessed for their adhesive activity, both Thbs4−/− and mutTSP-4 KI EC adhered poorly compared with WT EC (Figure 7B).

Quantification of the Trypan Blue staining of cells attached to the cell culture plates coated with fibronectin or fibronectin mixed with r-TSP-4 or r-mutTSP-4 resulted in similar differences between experimental conditions (Figure IIA and IIB in the online-only Data Supplement), although quantification of DNA appeared to be more sensitive in detecting the differences between cells in different conditions.

Discussion

Our results identify TSP-4 as a novel regulator of angiogenesis. TSP-4 is a member of the thomboospondin family that includes 4 other proteins: TSP-1, TSP-2, TSP-3, and TSP-5 (COMP).6,8 TSP-1 and TSP-2 have evolved more recently and are potent antiangiogenic proteins.16,40–43 Their antiangiogenic properties have been documented in vitro and in vivo and are attributed to the TSP repeats where the binding site for the cell receptor CD36 and the sequence involved in regulation of matrix metalloproteinase activity reside.44,45 TSP-3 and TSP-4, and TSP-5 belong to the more ancient TSP subgroup,8 and they have not been reported to be regulators of angiogenesis. None of the latter 3 TSPs has the TSP repeats domain associated with the antiangiogenic activity of TSP-1 and TSP-2.6,17,46

TSP-4 has attracted significant interest recently and has been associated with the remodeling of vasculature and myocardium,9,12,21,22,47 control of the organization and function...
of tendon and skeletal muscle, risk for the cardiovascular disease, inflammation, and synaptogenesis in the central nervous system. The existing data document the association between TSP-4 and cancer, especially with the breast cancer. However, the mechanisms of these associations remain unknown.

In this report, we describe a novel and unexpected function of TSP-4: stimulation of angiogenesis. We documented the proangiogenic activity of TSP-4 in several complementary models: in vivo Matrigel angiogenesis, in vivo tumor angiogenesis, postnatal retinal vasculature development model, skin wound healing, and in several in vitro assays, including cultured EC adhesion, migration, and proliferation assays. Although the deficiency in TSP-4 resulted in decreased angiogenesis or reduced proangiogenic functions, both r-TSP-4 and A387P r-mutTSP-4, which is more active in its cellular interactions, increased proangiogenic properties in vitro and both stimulated angiogenesis. Throughout the present study, we did not observe any sex-specific effects of either TSP-4 or mutTSP-4 P387 on angiogenesis. Although we did not systematically examine the age-dependence of the effects, our results indicate that the effect of TSP-4 on angiogenesis can be detected at different ages, from 5-day-old pups (postnatal retinal vasculature development) to 27-week-old mice (Matrigel plug assay).

The in vivo Matrigel plug assay is a simple and convenient model to study the growth of new blood vessels in the absence of influences from surrounding tissue. Angiogenesis was evaluated by the levels of 3 angiogenesis-related markers (CD31, a marker of EC; α-actin, a marker of smooth muscle cells/pericytes and of maturation of the growing capillaries; and laminin-1, a marker of basement membrane). Angiogenesis was reduced in Thbs4−/− mice, indicating that TSP-4 stimulates angiogenesis. TSP-4 was detected in growing blood vessels, but its localization was different compared with its localization in mature blood vessels where TSP-4 was present on the abluminal side of the EC monolayer in the vascular wall. In new vessels growing in the Matrigel plug, TSP-4 was detected in the lumen. This localization suggests that TSP-4 may be secreted by EC or pave a path for EC to form the new vessel. Although TSP-4 was found in a proximity to EC in glowing vessels, we did not address its source systematically. Smooth muscle cells can be producing TSP-4 as we reported in the past, and the blood cells as a source of TSP-4 cannot be excluded although we did not detect any production of TSP-4 protein by the blood cells (data not shown), and others never reported its production by the blood cells; we did detect TSP-4 mRNA in the cellular fraction of blood (data not shown).

In the mouse breast cancer angiogenesis model, host TSP-4 deficiency alone did not reduce tumor mass or the levels of the angiogenesis markers. We found that the cancer cells produced TSP-4 in vivo. TSP-4 is a secreted protein that becomes available to all cell types after it is released. Therefore, we considered whether TSP-4 produced by the cancer cells could have provided sufficient signals to EC and other vascular cells to maintain angiogenesis within the tumors. When we used EMT6 stably transduced with lentiviral particles expressing TSP-4 shRNA that did not produce TSP-4 in the in vivo cancer angiogenesis model, the weights of tumors were significantly decreased in the absence of TSP-4 as compared with the weights of tumors in WT mice. The levels of angiogenesis markers were also significantly decreased, indicating that TSP-4 facilitates tumor angiogenesis and growth. The source of TSP-4 (vascular- or cancer-cell–produced) does not seem to be important, rather the level of TSP-4 in tumors seems to be sufficient to support tumor angiogenesis and growth based on the lack of the effect in our experiments that used WT EMT6 in Thbs4−/− mice.
A387P TSP-4 has been associated with cardiovascular disease in several patient cohorts. We have reported that A387P TSP-4 is more active in interactions with cells and produces more pronounced effects in cell culture. Thus, the enhanced angiogenesis and cancer growth observed in the KI mice expressing A387P TSP-4 compared with WT TSP-4 is consistent with its increased bioactivity.

Wound healing is a process of tissue remodeling and is dependent on angiogenesis. In skin wounds, angiogenesis is clearly detected by day 3 and is active until at least day 7. The healing at this stage is accelerated if angiogenesis is increased and delayed if angiogenesis is inhibited. We have tested Thbs4−/− mice in an excisional skin wound healing assay. Healing was significantly delayed in Thbs4−/− mice, consistent with the proangiogenic effects of TSP-4 in other models. The delay was detectable on the day 3 and remained statistically significant until day 7. Wound healing was accelerated in mutTSP-4, once again consistent with the greater activity of P387 TSP-4 in cellular and physiological processes.

Each angiogenesis model examined has advantages and limitations. The Matrigel model allows analyses of the formation of new vessels in the absence of surrounding tissue, but the plug induces an inflammatory response in the host. The cancer angiogenesis models involve complex interactions of the vascular cells with the surrounding tissue, but the vessels formed in a tumor are different from the vessels developing during the normal physiological processes of remodeling. The outcomes in the skin wound healing model depend not only on angiogenesis, but also on an inflammatory response and the function of fibroblasts and keratinocytes.

The postnatal retinal development model is another way to address the effects of protein knockout or overexpression on angiogenesis. In this model, the angiogenesis can be observed in the absence of proinflammatory signals and external interventions. In this model, TSP-4 knockout resulted in delayed development of retinal vasculature, whereas mutTSP-4 accelerated the process.

In the in vivo models that we used to study the role of TSP-4 in angiogenesis, TSP-4 knockout and mutTSP-4 expression in KI mice produced opposite effects that we could expect based on our previous studies of the effects of TSP-4 and mutTSP-4. Although the effects of compensation can never be completely excluded when working with transgenic mice, the effects of compensation seem to be less likely when the knockout of a protein and an overexpression of a mutant protein with a known increased activity result in opposite and predicted effects. We previously examined the expression and localization of TSP-3 and TSP-5, 2 proteins highly homologous to TSP-4, in tissues and blood vessels of Thbs4−/− mice, and we found that their expression was not changed and that they displayed a distinct nonoverlapping localization in tissues.

Our in vitro experiments performed using EC from Thbs4−/− and mutTSP-4 KI mice and recombinant TSP-4 and mutTSP-4 complement and support the conclusions obtained in the in vivo models. Although the in vitro approach had its limitations (eg, the source of TSP-4 may affect its function, EC are studied in isolation from other cell types, etc.), it complemented the in vivo approach, lead to similar conclusions, and allowed to begin to investigate the cellular mechanisms of the observed effects.

Invasion of EC into tissue and formation of a vessel lumen is a multistep process. A variety of activities of EC and extracellular matrix proteins are involved in this complex response. We have examined the effect of TSP-4 and A387P TSP-4 on EC adhesion to the matrix, EC proliferation, and EC migration. In all 3 in vitro models, we observed an effect of TSP-4 consistent with its proangiogenic activity. r-TSP-4 increased EC proliferation, whereas the proliferation was inhibited in Thbs4−/− EC. Thbs4−/− EC demonstrated decreased adhesion, although r-TSP-4 did not have any effect on WT EC adhesion. Differences in the effects of endogenous and exogenously introduced TSP is a known phenomenon: the effects of a TSP on cells greatly depends on its origin (endogenous versus exogenous).

TSPs have multiple protein ligands that modify their effects, and some of the binding events occur during the production and secretion of TSPs. r-TSP-4 increased the migration of EC, and the migratory capacity was reduced in EC from Thbs4−/− mice.

The effects of r-mutTSP-4 were more complex in the in vitro assays. r-mutTSP-4 increased EC migration, and EC from mice expressing mutTSP-4 demonstrated increased migration as well. Adhesion was inhibited by r-mutTSP-4, both in the in vitro experiments with the recombinant mutTSP-4 and with EC from mutTSP-4 KI mice. This observation is consistent with our previous report on the effect of r-mTSP-4 on human EC. Although r-mutTSP-4 increased EC proliferation, there was a significant decrease in proliferation of mutTSP-4 KI EC. This discrepancy may be because of a complexity of the mutTSP-4 effects on EC that are not fully reproduced in an in vitro proliferation assay or to a compensation of function(s) in the transgenic mouse. In either case, we conclude that mutTSP-4 may acquire additional properties compared with WT TSP-4 rather than just being more potent in cellular interactions.

In sum, we have identified a novel function for TSP-4: its capacity to regulate angiogenesis. TSP-4 is a new proangiogenic ECM protein. It influences multiple EC responses in vitro that translate into enhanced blood vessel formation in vivo.

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Disclosures
None.

References


**Significance**

Thrombospondin-4 (TSP-4) was found to be abundantly expressed in vasculature and in several cancers and to play an important role in tissue remodeling. However, the significance of its expression in blood vessels and its effect on angiogenesis remained unknown. The TSP protein family has been associated with regulation of angiogenesis, but the effects of TSP-1 and TSP-2, 2 known angiogenesis regulators, are antiangiogenic. The differences in the structure of TSP-1/TSP-2 and TSP-4 suggest that they may have distinct or even opposite functions in vasculature. The results of our work described in this report revealed that TSP-4 is a novel proangiogenic ECM protein that promotes EC adhesion, migration, and proliferation and increases angiogenesis in in vivo Matrigel and cancer models. Thus, we have identified a novel protein that promotes angiogenesis.
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Materials and Methods

**Cell isolation and culture:** Mouse Lung Endothelial Cells (EC) were isolated from wild-type (WT), Thbs4−/−, and mutant A387P TSP-4 knock-in (mutTSP 4 KI) mice as described by Mahabaleshwar and colleagues. EC were cultured in DMEM/F-12 medium (Sigma) supplemented with 20% fetal bovine serum (HiClone), 80 mg/L heparin (Sigma), and 40 mg/L endothelial cell growth supplement (ECGS) purified from bovine brain tissue. Cells were grown in 6-, 12- or 24-well clusters until they became confluent.

The EMT6 mouse breast cancer cell line was purchased from ATCC (Manassas, VA) and grown according to the ATCC recommendations until harvested.

**Adhesion assay:** Adhesion assays were performed as previously described. The cultured EC were trypsinized, counted, and 5x10^5 cells were added in complete medium with 5% serum to the wells of 24-well plates (CoStar, Corning, New York, #3526) pre-coated with fibronectin (Sigma Aldrich, St. Louis, Missouri, #F1141) along with recombinant TSP-4 (r-TSP-4) or recombinant A387P TSP-4 (r-mutTSP-4) overnight at 4°C. r-TSP-4 and r-mutTSP-4 were added to the coating solution at the concentration 10 µg/ml as was done previously and fibronectin was used at the concentration 10 µg/ml. Plates were incubated for 1 h at 37 °C, then washed with PBS once and stored at -80 °C for 3 h. After thawing the plates, CyQuant reagent (Invitrogen, Grand Island, New York, #C35007) was used to quantify cell DNA in the wells.

**Migration assay:** Migration assays were performed using trans-well chambers (Fisher, Corning, New York, #07200149). The bottom of the trans-well chambers were coated with r-TSP-4 or r-mutTSP-4 overnight at 4 °C. EC (10^6 cells) were resuspended in the serum-free DMEM, transferred to the trans-well chambers, and incubated at 37 °C for 4 hours. Complete EC medium with 20% serum was used as a chemoattractant. To identify the receptors for TSP-4 involved in EC migration on TSP-4 substratum, we used the following function blocking antibodies: anti-integrin β1 antibody (Millipore, #MAB2253Z), anti-integrin β3 antibody (Millipore, #MAB2023Z), anti-integrin αvβ3 antibody (Millipore, #MAB1976Z), anti-integrin αvβ5 antibody (Millipore, #MAB1961Z), anti-integrin α2 antibody (Millipore, #MAB1950Z), and anti-CD47 antibody (Millipore, #ab3283). Gabapentin (Sigma, #G-007) was used to block TSP-4 interaction with gabapentin receptor αvβ3. After plating, the cells were treated with the selected antibodies (20 µg/ml) and incubated at 37 °C for 4 hours. After 4 hours, the medium was aspirated, and cells were removed from the inside surface of the upper chamber using cotton swabs. The plates were frozen in -80 °C for 3 hours, and the DNA of migrated cells was quantified using CyQuant reagent.

**Proliferation assay:** Proliferation assays were performed in 12-well cell culture plates (CoStar, St. Louis, Missouri, #3513) coated with fibronectin with or without added r-TSP-4 or r-mutTSP-4 as described for adhesion assays. 5,000 cells were plated per well in complete DMEM medium with 5% serum, and cells were left to grow for 4, 24, 48 and 72 h. At each time point, the wells were washed with PBS once and stored at -80 °C for at least 3 h, and then DNA in the wells was quantified using CyQuant reagent.

**Recombinant TSP-4:** recombinant WT TSP-4 (r-TSP-4) and r-mutTSP-4 bearing the A387P substitution were purified from the culture media of mammalian cells stably transfected with THBS4 cDNA as previously described.

**Animals:** C57/BL6J male and female mice (Jackson Laboratories) were used in the described experiments (n≥10 per experimental point or condition as specified in the
Figure Legends). Thbs4−/− mice were described previously. A387P TSP-4 knock-in mice (mutTSP-4 KI) were developed at Wyeth Pharmaceuticals. To create the A387P TSP-4 knock-in (mutTSP-4 KI) mouse, a point mutation was introduced in a genomic clone spanning 8-10 exons, the neo cassette was introduced into the construct, and the targeted mutant construct was electroporated into embryonic stem cells. These mice express P387 TSP-4 under the endogenous TSP-4 gene promoter (only a fragment of DNA where the SNP was found was substituted). The homology between human and mouse TSP-4 is close to 100%. Normally, the mouse TSP-4 gene codes for A387 TSP-4; thus, we decided to create a P387 TSP-4 KI mouse to explore the functional consequences of the SNP variation in vivo. Based on our results with human TSP-4 and the very high evolutionary conservation of the protein, such substitution in the mouse gene should result in consequences similar to the ones seen in human TSP-4. P387 TSP-4 is expressed in the same pattern as the naturally occurring A387 variant in WT mice (data not shown). The KI mouse colony has been maintained for more than 5 years in C57Bl/6 background. The mice develop and reproduce normally, and do not have an overt phenotype without challenge. Animals were housed and cared for in the AAALAC-approved animal facilities of the Cleveland Clinic. All animal studies were approved by the Institutional Animal Care and Use Committee, and all experiments were conducted in strict accordance with the National Institutes of Health and institutional guidelines. Ketamine (80 mg/kg)/Xylazine (5 mg/kg) mixture was used for anesthesia to immobilize the mice for subcutaneous Matrigel or cancer cell injections.

**Matrigel plug angiogenesis assay:** Twenty-six- to twenty-seven-week-old mice were anesthetized by IP injection of Ketamine (80 mg/kg)/Xylazine (5 mg/kg) mixture, and the neck area was shaved and swabbed with 70% ethanol. Mice were injected subcutaneously in the upper dorsal side with 750 µL of Matrigel Matrix Basement Membrane (BD Biosciences) supplemented with bFGF (750 ng/mL, R&D Systems) and heparin (26 U/mL, Sigma). Subcutaneous plugs were removed 7 days later and frozen in OCT in liquid nitrogen. Sections (10 µm) of the plugs were stained using antibodies against markers of blood vessels as described using antibodies against CD31, laminin-1, and α-actin.

**Cancer angiogenesis assay:** Fifteen- to sixteen-week-old mice were anesthetized by IP injection of Ketamine (80 mg/kg)/Xylazine (5 mg/kg) mixture. Mice were injected with 1.5 x 10^6 EMT6 cells in 100µl volume of PBS into the mammary fat pad. Ten days later, tumors were excised and frozen in OCT in liquid nitrogen. The characterization of angiogenesis in tumor sections was performed as described for Matrigel plugs.

**Transduction of EMT6 cells with lentiviral particle containing shRNA for TSP-4 and development of EMT6 stably expressing TSP-4 shRNA:** EMT6 cells were plated in 6 well plates and grown according to ATCC recommendations. After 24 h, cells were transduced with 10,000 IFU/ml lentiviral particles containing TSP-4 shRNA (Santa Cruz Biotechnology, Dallaz, TX) as per the manufacturer’s directions. After 24 h, the transduction medium was changed to the growth medium for an additional 48 h. The cells were then treated with 10 µg/ml puromycin for 2 weeks. The cells expressing the construct were selected and maintained for the in vivo cancer angiogenesis experiments.

**Retinal vasculature development in flat-mounted whole retinas** was assessed in 5-day-old Thbs4−/−, mutTSP-4 KI, and C57Bl/6 mice by staining of endothelial cells with the Alexa568-conjugated lectin (Isoslectin GS-IB4 From Griffonia simplicifolia, I21412, Life Technologies) essentially as described elsewhere. Briefly, the 5-day-old pups were
euthanized by CO₂ exposure. Eyes were removed and fixed in 4% paraformaldehyde for 30 min. Corneas were excised, lenses and vitreous were removed, retinas were dissected out and post-fixed for additional 2 hrs without flattening. Retinas were blocked and permeabilized in 1% BSA/5% goat serum with 0.5% Triton X-100 in PBS overnight, washed x3 in PBS, and incubated with GS-IB4-A568 (0.02 µg/µl in PBS containing 1% BSA/5% goat serum) overnight. The retinas were washed in PBS and flattened by four radial insisions, placed on a glass slide and mounted with Vectashield mounting media (Vector Labs). An image of each whole retinal flatmount was obtain by taking 9 overlapping microphotographs using Zeiss Axio Imager.Z1 with Plain Apochromat 5x objective, AxioCam MRC5 camera and AxioVision v4.8.2 software followed by merging corresponding microphotographs into one by using Adobe Photoshop CS5 (Adobe Systems).

Quantitative vascular analysis: Analysis of retinal vasculature in flat-mounts stained with Alexa-568-labeled GS-IB4 lectin was performed using customized, automated algorithms and scripts written for ImagePro Plus (v7.0, Media Cybernetics, Silver Spring, MD). Briefly, for each retinal image, the tissue boundary (i.e. Region of Interest, ROI) was segmented using a combination of low-pass and connected components algorithms to provide a measure of Total Retinal Area. Subsequently, a "flattening" filter was applied to compensate for any unevenness in the image resulting from lens distortion. The ROI for vessel extension from the optic nerve was then delineated using a combination of intensity thresholding, morphological "closing" operations, and largest connected component analysis. This area within this ROI was calculated and exported as the Vessel ROI Area. To segment vessels in the retina, the vasculature in the processed lectin images was enhanced and equalized using spectral filters and subsequently thresholded to generate a binary vessel mask. Vessel Area was then calculated by summing pixels in this mask. Similarly, the Vascular Length parameter was calculated by summing pixels in this mask after "skeletonization" or thinning of vessels to a 1-pixel thick midline representation. Using this skeletonized vessel mask, the 8-pixel neighborhood around each skeletal pixel was examined; any pixel with 3 or more skeletal pixels in its neighborhood (constituting a vessel branching point) was summed to provide the Total Nodes in the retina. Lastly, using the mask generated for Vessel ROI Area analysis (vessel extension mask), 360 vectors were extended from the center of the optic nerve to the vessel mask periphery (1 degree per rotation). For each of these vectors, the distance to the edge of the vessel periphery was calculated, summed and divided by 360 to derive Mean Vessel Extension. To enable visual verification of algorithm performance, a pseudo-colored overlay was generated for each image analyzed. This overlay image is comprised of a grayscale representation of the original lectin image in which a yellow outline delineates the segmented retinal tissue, a blue outline delineates the segmented vessel extension ROI, the skeletonized segmented vasculature is indicated in red, and green pixels represent the vascular branch nodes.

Skin wound healing: Excisional wounds were made using the Acu-Punch (Acuderm Inc., Ft. Lauderdale, Florida, #CE0403), scissors, and forceps. Wound pictures were taken, and the area of the wounds was measured on the day of surgery and on days 1, 3, 7, 10, 11, 12, 13, and 14 followeing the surgery. The images of the wound area were analyzed and quantified using Adobe Photoshop software.

Immunohistochemistry: Sections of Matrigel plugs, skin, and tumors were processed as previously described 5, 8, 10, 12 and stained using Vecta Stain ABC Kit according to the manufacturer's instructions to detect the expression of angiogenesis markers. EC were
visualized in Matrigel plug sections using anti-vWF antibody (Accurate Chem, NY), and Biotinylated rat anti-mouse CD31 (BD Biosciences, 1:100), rabbit polyclonal anti-\(\alpha\)-actin (Abcam, 1:200), and rabbit polyclonal anti-laminin-1 (Abcam, 1:300) were used to visualize EC, smooth muscle cells/pericytes, and basement membrane protein, respectively. Goat monoclonal anti-TSP-4 (R&D systems; 1:100) was used to examine TSP-4 protein expression. Visualization after staining with antibodies was performed using a high-resolution slide scanner (Leica SCN400FL, Leica microsystems, GmbH, Wetzlar, Germany) at 20X magnification. High-resolution images of whole sections were generated and quantified to determine the percentage of the stained area and the intensity of staining using ImagePro 6.1 software.

**Statistical analysis:** The analyses of the data were performed using the programs in Sigma Plot Software: one-way ANOVA was used to determine the significance of parametric data, and the Wilcoxon test was used for non-parametric data sets. The significance level was set at \(p<0.05\), and the significant differences in experimental groups is marked in the figures. Error bars in all figures represent SEM. The number of mice or mouse retinas in each group or the number of independent \textit{in vitro} experiments is indicated in Figure legends as \(n\).
References.


Supplemental Figure I: A: MLEC were added to 24-well plates and incubated at 37 °C for 48 h. Unattached cells were removed by washing, and the remaining cells were fixed with 4% paraformaldehyde, stained with Trypan Blue, and counted using Adobe Photoshop software. A: MLEC from WT mice were added to the plates coated with fibronectin (control) or fibronectin mixed with rTSP4 or mTSP4. B: EC isolated from WT, Thbs4−/−, and mutTSP4 KI mice were plated onto fibronectin-coated plates. *p<0.05 compared to WT, n=3.
Supplemental Figure II: MLEC were added into 24-well plates and incubated at 37 °C for 1 h. Unattached cells were removed by washing, and the remaining cells were fixed with 4% paraformaldehyde, stained with Trypan Blue, and counted using Adobe Photoshop software. A: MLEC from WT mice were seeded into the plates coated with fibronectin (control) or fibronectin mixed with rTSP4 or mTSP4. B: EC isolated from WT, Thbs4−/−, and mutTSP4 KI mice were used. *p<0.05 compared to WT cells, n=3.