Thrombospondin-1 Is a Plasmatic Marker of Peripheral Arterial Disease That Modulates Endothelial Progenitor Cell Angiogenic Properties


Objective—We examined whether plasma levels of angiogenic factors are altered in plasma of patients with peripheral arterial disease (PAD) and whether these factors affect endothelial progenitor cell–induced angiogenesis.

Methods and Results—Plasma was collected from 184 patients with PAD and 330 age-matched healthy controls. Vascular endothelial growth factor and placental growth factor concentrations did not differ between the groups, whereas we found a linear correlation between PAD disease and thrombospondin (TSP)-1 plasma level. TSP-1 was expressed in newly formed vessels in PAD patients having received local injections of bone marrow mononuclear cells. To analyze the functional role of TSP-1 during neoangiogenesis, we used a Matrigel-plug assay and showed that vascularization of implanted Matrigel-plugs was increased in TSP-1−/− mice. Moreover, injections of TSP-1 in C57Bl/6J mice after hindlimb ischemia induced a significant decrease of blood flow recovery. To investigate the effects of TSP-1 on human endothelial colony-forming cell (ECFC) angiogenic potential, recombinant human TSP-1 and a small interfering RNA directed against TSP-1 were used. In vitro, TSP-1 N-terminal part significantly enhanced ECFC adhesion, whereas recombinant human TSP-1 had a negative effect on ECFC angiogenic potential. This effect, mediated by CD47 binding, modulated stromal cell–derived factor 1/CXC chemokine receptor 4 pathway.

Conclusion—TSP-1 is a potential biomarker of PAD and ECFC-induced angiogenesis, suggesting that TSP-1 modulation might improve local tissue ischemia in this setting. (Clinical trial registration: NCT00377897.) (Arterioscler Thromb Vasc Biol. 2011;31:551-559.)

Key Words: angiogenesis ■ arterial thrombosis ■ endothelial progenitor cells

Peripheral arterial disease (PAD), characterized by atherosclerosis of the lower extremities, affects up to 15% of people older than 55 years.1 The main clinical manifestations of PAD are intermittent claudication and critical limb ischemia (CLI). Intermittent claudication is characterized by reproducible pain on exertion that is relieved by rest. CLI is the most severe form of PAD and is characterized by the inability of arterial blood flow to meet the metabolic demands of resting muscle or tissue, resulting in rest pain and/or tissue necrosis and frequently necessitating amputation. Currently, PAD diagnosis is based on the ankle–brachial pressure index (ABI), but the ABI is a poor marker of PAD severity. There are no other reliable diagnostic tests for PAD, and new biomarkers would therefore be useful.

Atherosclerosis induces occlusion of the arterial tree and tissue hypoxia, which is a strong stimulus for angiogenesis. Collateral vessels develop physiologically in patients with CLI, mainly driven by an enhanced angiogenic response. However, the capacity of this compensatory mechanism is rapidly exceeded, and normal flow is not restored. Autologous endothelial progenitor cells (EPCs) are candidates for angiogenic therapy. Because of their scarcity in human samples, EPCs have been characterized by culture methods. At least 2 populations of EPCs have been described.3 “Early” EPCs appear within 4 to 7 days of culture, whereas “late” EPCs, also called endothelial colony-forming cells (ECFCs), develop after 2 to 3 weeks and have the characteristics of precursor cells committed to the endothelial lineage. We have
previously shown, in amputation specimens from patients with CLI who had received local therapeutic injections of bone marrow mononuclear cells (BM-MNCs), that endothelial cells from newly formed vessels have an ECFC phenotype. This is in keeping with results obtained with preclinical models showing that ECFCs are the cell type responsible for blood vessel formation.

A number of angiogenic growth factors may modulate vascular growth and EPC angiogenic potential during PAD. Vascular endothelial growth factor (VEGF) is probably one of the most important proangiogenic factors. Elevated VEGF plasma levels were found in a study of 46 PAD patients. Placental growth factor (PIGF), which is not required for normal embryonic vascular development, could also play a critical role in pathological angiogenesis. Anti-angiogenic factors could likewise be involved in PAD. Thrombospondin (TSP)-1 has been found to be overexpressed in histological sections of human and mouse ischemic leg tissues. However, plasma TSP-1 levels have not yet been measured in PAD patients.

Here, we compared concentrations of the circulating angiogenesis-related factors VEGF, PIGF, and TSP-1 in 184 PAD patients enrolled in the PALLAS clinical study and in 330 paired healthy controls. Because TSP-1 levels were higher in the patients than in the controls, we further investigated the effect of TSP-1 on the ECFC angiogenic properties.

Methods

Study Population for Plasma Concentrations of VEGF, PIGF, and TSP-1

PAD patients (n=184) were enrolled in our vascular medicine department over a 2-year period. Patients were eligible if they were white men younger than 70 years with symptomatic atherosclerotic disease of the lower limbs and an ankle–brachial systolic pressure index (ABI) <0.90, or a history of surgical or endovascular revascularization, as previously reported. Age-matched control subjects (n=330) with no history of arterial disease were randomly selected as described previously.

Local Injection of BM-MNCs in PAD Patients With CLI

The OPTIPEC clinical trial was a phase I nonrandomized study. Briefly, patients were eligible for the protocol if they had CLI associated with limited gangrene or a nonhealing ischemic ulcer and if they were not eligible for surgical revascularization or percutaneous angioplasty, or if such a procedure had little chance of success. The cell therapy protocol was similar to that initially published by Tateishi-Yuyama et al.

ELISA Assays, ECFC Culture, In Vitro Angiogenesis Assays, In Vivo Matrigel-Plug Assay, and Hindlimb Ischemia Model

Experiments are described in detail in the online Data Supplement at http://atvb.ahajournals.org.

Results

Angiogenic-Related Factors in PAD

The baseline demographic and clinical characteristics of the study population are described elsewhere. Briefly, smoking, hypertension, diabetes, serum lipids, lipid-lower therapy, and glucose level were significantly different between patients with PAD (n=184) and controls (n=330) (Table I in the online Data Supplement).

Plasma concentrations of VEGF, PIGF, and TSP-1 were determined in patients and controls. VEGF and PIGF levels did not significantly differ between cases and controls (Figure 1A and 1B), whereas TSP-1 levels were significantly higher in PAD patients (Figure 1C; P<0.0001). After categorization in tertiles of plasma levels of VEGF, PIGF, and TSP-1, we found a U-shaped relationship between PAD and VEGF or PIGF plasma levels (supplemental Table II). Indeed, we found that upper and lowest tertiles were associated with disease. Concerning TSP-1, we found a linear correlation between occurrence of PAD disease and TSP-1 plasma level. Similar results were found after adjustment for common PAD risk factors (hypertension, hypercholesterolemia, diabetes, and smoking status), lipid-lowering treatment, and C-reactive protein. We next assessed whether common PAD risk factors affected VEGF, PIGF, and TSP-1 concentration. VEGF and PIGF significantly correlated with C-reactive protein. In addition, PIGF correlated with age, hypertension, and antiplatelet therapy. TSP-1 plasmatic levels were not associated with clinical parameters (supplemental Table III). Whereas TSP-1 levels were associated with the occurrence of PAD, we found no significant difference in plasma levels of VEGF, PIGF, and TSP-1 according to disease severity (supplemental Table IV).
TSP-1 Negatively Regulates Angiogenesis in Matrigel-Plug and Hindlimb Ischemia Models

To analyze the functional role of TSP-1 during neoangiogenesis, we used a Matrigel-plug assay in TSP-1−/− and C57Black6/J wild-type mice for 14 days. Plugs from TSP-1−/− animals (Figure 3A) had a higher blood vessel content than those from wild-type animals (Figure 3A). This was further confirmed by measuring the hemoglobin content of the plugs (Figure 3B; \(P=0.01\)). We have also determined the effect of 10 \(\mu g/mL\) TSP-1 added to the plugs implanted in wild-type and in TSP-1−/− mice. Figure 3C and 3D shows that TSP-1 induced a strong decrease in plug vascularization in both wild-type and TSP-1−/− mice (\(^*P=0.017\) and \(^*P=0.016\) respectively).

The hindlimb ischemia model in TSP-1−/− mice has been previously reported by Brechot et al, who found that TSP-1−/− mice were clinically and histologically protected from necrosis compared to wild-type controls. Tissue protection was associated with increased postischemic angiogenesis and muscle regeneration. In the present work, we have explored the effects of recombinant human TSP-1 in the model of hindlimb ischemia induced in C57Bl/6J mice by femoral artery and vein excision. As shown in Figure 3E and 3F, intramuscular injections of recombinant human TSP-1 induced a significant decrease in blood flow recovery 14 days after surgery (\(^*P=0.04\)).

Figure 2. TSP-1 are expressed in newly formed vessels after cell therapy product in peripheral arterial disease. TSP-1 was expressed by endothelial cells (black arrows) and macrophages (black arrowheads) in amputation specimens from CLI patients treated (C) and untreated (A) with local injections of BM-MNCs. Scale bar=50 \(\mu m\). Macrophage CD68-positive cells are shown in serial sections of amputation specimens from CLI patients treated (D) and untreated (B) with local injections of BM-MNCs. Scale bar=50 \(\mu m\).

TSP-1 Inhibition in ECFCs Induces a Proangiogenic Phenotype In Vitro and In Vivo

Because postnatal vasculogenesis is thought to involve EPCs, and given the expression of TSP-1 found in newly formed vessels in human, we further explored whether TSP-1 inhibition was able to modulate ECFC angiogenic potential. We focused on ECFCs because this cell type is currently proposed as the cell type at the origin of newly formed vessels. ECFCs express endothelial markers such as CD146, but they do not express leuko-monocytic markers such as CD14 and CD45. We found that ECFCs expressed a high surface density of the TSP receptor CD47, whereas no expression of CD36 was observed (supplemental Figure 1).

We verified that TSP-1 mRNA and protein expression remained strongly inhibited by the specific small interfering (si)RNA during the days following transfection in cord and adult blood ECFCs (supplemental Figures II and IV). We then explored the effect of TSP-1 inhibition on the angiogenic properties of ECFCs in vitro. TSP-1 inhibition induced a strong increase in ECFC proliferation (Figure 4A; \(P<0.0001\)), associated with a 4-fold increase in the gene expression of the nuclear proliferation marker Ki67 (Figure 4B; \(P=0.03\)). TSP-1 inhibition also induced ECFCs to adopt a proangiogenic phenotype, as reflected by enhanced pseudotube formation in Matrigel (Figure 4C).

TSP-1 Inhibition in ECFCs Upregulates Stromal Cell-Derived Factor 1/CXC Chemokine Receptor 4 Pathway

To examine the possible transcriptional effect of TSP-1 inhibition, we used real-time quantitative polymerase chain reaction to measure mRNA levels of several angiogenic factors and their receptors, including VEGFA, angiopoietin-2, stromal cell–derived factor (SDF)-1, and endothelial nitric oxide synthase. We also verified that TSP-1 inhibition did not modify TSP-2 expression. After 48 hours of transfection, TSP-1 inhibition resulted in a significant 2-fold increase in SDF-1 mRNA and a 4-fold increase in its receptor CXC chemokine receptor (CXCR)-4 mRNA (Figure 4D). Increased ECFC surface expression of CXCR4 was shown by flow cytometry (supplemental Figure III). This upregulation, first observed in cord blood–derived ECFCs, was also found in adult blood ECFCs, but not in human umbilical vein endothelial cells (supplemental Figure IVC). To explain the effect of TSP-1 inhibition on ECFC differentiation in pseudotubes and to explore the potential involvement of the SDF-1-CXCR4 pathway, we used 12G5, a monoclonal antibody recognizing an epitope located in the second extracellular loop of CXCR4 that we had previously used to reduce the angiogenic potential of ECFCs related to the
SDF-1/CXCR4 pathway. The increase in Matrigel tube formation induced by TSP-1 inhibition was abrogated by monoclonal antibody 12G5 (P=0.0007; Figure 4E). Finally, we tested TSP-1–inhibited ECFCs in a nude mouse model of hindlimb ischemia. ECFCs transfected with TSP-1 siRNA or control siRNA were intravenously injected into nude mice, 6 hours after femoral artery ligation. On day 7, foot perfusion was improved significantly more by TSP-1–inhibited ECFCs than by control ECFCs (P=0.03; Figure 4F).

TSP-1 N-Terminus Mediates ECFC Adhesion
We chose to test the effect of recombinant human TSP-1 on ECFC angiogenic properties. In keeping with reports that TSP-1 acts as an adhesion molecule, ECFC adhesion was significantly enhanced by recombinant human TSP-1 (Figure 5A). TSP-1 N-terminus has been described to be responsible for proangiogenic function of TSP-1. We thus compared the effect of TSP-1 to that of A1 peptide (TSP Hep I), a short peptide derived from the N-terminal part of TSP-1,19,20 A1 peptide induced a strong increase in ECFC adhesion, similar to that observed with TSP-1 (Figure 5A).

TSP-1 Decreases ECFC Proliferation and Pseudotube Formation by an N-Terminal–Independent Mechanism
To examine TSP-1–proliferative effect, ECFCs were plated in the presence or in the absence of precoated TSP-1 or A1 peptide. As shown in Figure 5B, TSP-1 significantly reduced ECFC proliferation, whereas no effect was observed with A1 peptide. TSP-1 inhibitory effect on proliferation could be explained in part by an increase of apoptosis, as reflected by a significant increase in proapoptotic protein p53 and FasL transcription after TSP-1 incubation (supplemental Figure V). The upregulation of these 2 pathways was previously proposed to explain antiangiogenic and/or antimetastatic properties of TSP-1.21–23 In an in vitro Matrigel model, an increase in pseudotube formation was observed with A1 peptide (Figure 5C and supplemental Figure VII), whereas TSP-1 significantly inhibited ECFC organization into branched structures and pseudotubes. This effect was further attributed to a downregulation of SDF-1 and CXCR4 (supplemental Figure VI).
CD47 Inhibition in ECFCs Induces the Same Proangiogenic Phenotype As TSP-1 Inhibition

The differences observed between TSP-1 pro- and antiangiogenic properties are probably the result of an imbalance between the proadhesive effect of the TSP-1 N-terminal part and another mechanism involving TSP-1 receptors. Because CD36 was not found on ECFCs, such as described previously in human umbilical vein endothelial cells (supplemental Figure 4.)

Figure 4. Inhibition of TSP-1 in ECFCs increases their angiogenic potential in vitro and in vivo. A, Inhibition of TSP-1 with siRNA promotes ECFC proliferation. The effect of TSP-1 siRNA on ECFC proliferation was evaluated by measuring the release of the paranitrophenol (pNP) (optical density [OD] at 405 nm) in EBM2 medium containing 5% FBS (mean ± SEM) (P < 0.001). B, Quantitative analysis of the proliferative nuclear antigen Ki67 mRNA by real-time quantitative polymerase chain reaction, after 72 hours of transfection with control siRNA (AllStars Negative Control; Qiagen) or TSP-1 siRNA (Santa Cruz Biotechnology). The mean and SEM of 3 experiments are shown (P < 0.03). C, siRNA-transfected ECFCs (3 × 10⁴) were seeded on Matrigel, and tube formation was measured by phase-contrast microscopy. D, Effect of TSP-1 inhibition with siRNA on the mRNA levels of VEGF, angiopoietin-2, and SDF-1 and their receptors. Values are expressed as means ± SEM and are the result of 3 independent experiments. E, Inhibition of pseudotube formation in response to TSP-1 inhibition with siRNA in ECFCs by monoclonal antibody 12G5. The mean and SEM of 3 experiments are shown. Quantitative analysis of network length was determined with Videomet software (Microvision). Values are expressed as means ± SEM and are the result of 3 independent experiments. F, Inhibition of TSP-1 in ECFCs increases their proangiogenic potential in hindlimb ischemia. Representative photomicrographs and quantitative evaluation of foot perfusion in mice injected with PBS or control siRNA– or TSP-1 siRNA–transfected ECFCs. Values are means ± SEM; n = 10 per group.
Figure 1).24 we inhibited CD47 gene expression in ECFCs. CD47 protein expression is strongly inhibited by the CD47 siRNA (Figure 6A). We explored the effect of CD47 inhibition on the angiogenic properties of ECFCs in vitro. ECFC adhesion was not modified by recombinant human TSP-1 between control siRNA– and CD47 siRNA–transfected ECFCs (Figure 6B). However, as for TSP-1 inhibition, CD47 siRNA induced a significant increase in ECFC proliferation (Figure 6C; \( P < 0.0001 \)) and pseudotube formation (Figure 6D and 6E; \( P < 0.0001 \)).

**Discussion**

Our findings suggest that the balance between angiogenic and antiangiogenic factors are altered in peripheral artery disease and that TSP-1 is associated with a loss of EPC angiogenic potential.

Plasma concentrations of TSP-1 in 184 patients with PAD were significantly higher than in controls, and we found a linear correlation along the different tertiles between PAD and TSP-1 plasma level. The increased TSP-1 plasma concentrations observed here may reflect an overexpression by ischemic tissues, as previously reported for cultured endothelial cells subjected to hypoxia.25 In line with this hypothesis, Favier et al previously showed by in situ hybridization an absence of TSP-1 labeling in control muscles by comparison to ischemic tissues of PAD patients and an intense TSP-1 labeling in the ischemic samples.11 Moreover, we found that newly formed vessels in patients treated with BM-MNCs were positive for TSP-1, pointing to an autocrine in situ effect during neoangiogenesis. However, it would be overstated to give any further conclusion about the role of TSP-1 in vessel function. Our aim was to discover an angiogenic biomarker potentially detectable in PAD patient plasma and to correlate it with the neoangiogenesis process in patients treated with a cell therapy product. One explanation is that treated patients who had to receive amputations had a neoangiogenic process inefficient to allow the vessel salvage. In this case, TSP-1 expression could represent a signal to prevent neoangiogenesis.

TSP-1 and TSP-2 are very similar structurally and functionally; both have been implicated as inhibitors of angiogenesis, endothelial cell survival, and endothelial cell migration.26 Loss of TSP-1 or TSP-2 during embryonic development is not lethal but results in increased vascular density.27,28 Double knockout for TSP-1 and -2 were shown to modulate proangiogenic activity of thrombopoietic cells.29 However, because of contradictory results about TSP-2 implication in ischemic injury response, we focused our work on TSP-1.30 TSP-1 is a heterogeneous molecule in terms of its cellular origin and functions, different parts of the molecule showing either pro- or antiangiogenic properties. Platelets may be an important source of circulating TSP-1 in PAD patients. Indeed, TSP-1 is stored in \( \alpha \)-granules and is released during platelet activation, an important phenomenon in PAD underlined by the efficacy of antiplatelet agents in this setting. During various pathological situations, and according to thrombin concentration level, platelets have been shown to release either their \( \alpha \)-granule–containing angiogenic factors or those containing antiangiogenic molecules31 or soluble CD40 ligand.32 Moreover, activated platelets33,34 or their

Figure 5. Recombinant human TSP-1 increases ECFC adhesion by its N-terminal extremity. A, Adhesion assays were performed in 24-well polystyrene plates (Becton-Dickinson) with or without recombinant TSP-1 or A1 peptide at 10 \( \mu \)g/mL. After incubation for 20 minutes, nonadherent cells were discarded, and the number of adherent cells was determined measuring the release of pNP (OD at 405 nm). Each data point is the average of 3 wells, and each experiment was performed at least 3 times. B, Proliferation assays were performed in 24-well plates with or without TSP-1 or A1 peptide at 10 \( \mu \)g/mL. After 72 hours, nonadherent cells were washed off, and the number of adherent cells was determined by measuring the release of pNP (OD at 405 nm). Each data point is the average of 3 wells, and each experiment was performed at least 3 times. C, ECFCs are plated in an 18-hour Matrigel tubule-formation assay, with or without TSP-1 or A1 peptide at 10 \( \mu \)g/mL. Quantitative analysis of network length was determined with Videomet software (Microvision). The mean and SEM of 3 experiments are shown.
derived microparticles have been shown to promote recruitment, differentiation, and/or angiogenic potential of progenitor cells, including also transfer of membrane receptors such as CXCR4. The role of TSP-1 in CLI has been explored in TSP-1 knockout mice, in which macrophages infiltrating ischemic tissues were found to have a less proinflammatory phenotype than those in wild-type mice. TSP-1 knockout led to enhanced tissue regeneration and prevented necrosis.

We investigated in vitro and in vivo the effect of TSP-1 on the angiogenic properties of cultured ECFCs, cells known to be involved in neoangiogenesis process. Interestingly, we found that TSP-1 had a double-edged effect on ECFCs, enhancing ECFC adhesion probably mediated by its N-terminal part, while reducing their proliferation and differentiation in pseudotubes, probably a consequence of TSP-1 binding to CD47. These 2 effects could contribute to the vessel phenotype in patients treated with BM-MNCs. TSP-1 is expressed in PAD ischemic tissues, to which it is thought to attract progenitor cells. Indeed, TSP-1 enhances the expression of adhesion molecules, and can also act via proteoglycans. TSP-1 also binds to CD36 and/or CD47, that have important roles in cell migration, proliferation or apoptosis. It has also been suggested that TSP-1 blocks the activation of matrix metalloproteinases such as matrix metalloproteinase 9, and thereby prevents the release of VEGF protein from extracellular stores. ECFCs were negative for CD36, whereas a strong expression of CD47 was observed. CD47 inhibition in ECFCs gave the same proangiogenic phenotype than TSP-1 inhibition. These results are in line with those from several studies that propose CD47 as a target to improve ischemia caused by aging or PAD.

However, adhesive properties of TSP-1 did not allow EPCs to form viable and normal vessels in patients with CLI who had received BM-MNCs. Indeed, newly formed vessels showed disorganized structures. In diabetic mice, TSP-1 mRNA expression by EPCs is significantly upregulated, whereas EPC angiogenic properties are downregulated in vitro and in vivo. We found that TSP-1 inhibition enhanced the ECFC angiogenic potential both in vitro and in vivo and that increased differentiation in pseudotubes could be attributed to SDF-1/CXCR4 pathway upregulation. In line with these observations, we show that vascularization of implanted Matrigel-plugs was significantly more efficient in TSP-1 mice than in their wild-type counterparts.

TSP-1 could potentially serve as a therapeutic target in PAD patients. The antiangiogenic effects of TSP-1 are classically attributed to the C-terminal domain, whereas its proangiogenic effects are attributed to its N-terminal domain. Peptides mimicking different parts of the TSP-1 molecule might modulate angiogenesis in PAD or be used to prime EPC or BM-MNC products, as reported with SDF-1. Moreover, the SDF-1/CXCR4 pathway upregula-

Figure 6. Inhibition of CD47 in ECFCs increases their angiogenic potential in vitro. A, Flow cytometry. CD47 siRNA decreased expression of CD47 on ECFCs. B, Adhesion assays were performed as described for Figure 5A, on control siRNA and CD47 siRNA, with or without recombinant TSP-1 at 10 ng/mL. No difference was observed on TSP-1 condition, for control siRNA- and CD47 siRNA–transfected ECFCs. C, CD47 siRNA promoted ECFC proliferation. The effect of CD47 siRNA on ECFC proliferation was evaluated by measuring the release of pNP (OD at 405 nm) in EBM2 medium containing 5% FBS (mean±SEM) (\(P<0.0001\)). D and E, Transfected ECFCs \((3\times10^5)\) were seeded on Matrigel, and tube formation was measured by phase-contrast microscopy. Quantitative analysis of network length was determined with VideoMet software (Microvision). The mean and SEM of 3 experiments are shown \((P<0.0001)\).
tion observed here after TSP-1 inhibition suggests that TSP-1 gene suppression could be used to produce an ECFC-based cell product effective in PAD. In addition, such an approach might be used to prevent restenosis after arterial interventions in diabetic patients. Alternately, pharmacological TSP-1 modulation in vivo might be beneficial in PAD. TSP-1 has also been reported to modulate blood flow in elderly subjects and in patients with atherosclerosis, by modifying NO levels. Drugs targeting TSP-1 might restore blood flow, enhance tissue healing, improve surgical outcomes, and reverse age-related vascular changes.

In conclusion, the increased plasma levels of TSP-1 found in PAD patients might contribute to the inadequate neovascularization observed in this setting. TSP-1 targeting ex vivo or in vivo might have the potential to modulate angiogenesis.

Acknowledgments

We thank Chantal Martin and all technicians from the animal facility of Institut Medicament-Toxicologie-Chimie-Environnement. We are indebted to the nursing service of Hoˆpital des Diaconesses (Paris) and Begin (Saint Mandé) for providing umbilical cord blood samples, and Dr Luiz Juliano for providing N-terminal domain of TSP-1. We thank Dr Jean-Luc Reny for patient inclusion in PALLAS study.

Sources of Funding

C.d’A. was supported by Fondation pour la Recherche Médicale. J.-V.D. received grants from the National Council for Scientific and Technological Development (CNPq, Brazil) and from Comissao de Aperfeicoamento do Pessoal de Nível Superior (CAPES). Centre National de la Recherche Scientifique pays the salary of C.B.-V. This work was supported by research grants from the Leducq Foundation, INSERM, and CAPES (Ministry for Education, Brazil)/COFECUB (France) (629/09). J.-V.D. received grants from the National Council for Scientific and Technological Development (CNPq, Brazil) and from Comissao de Aperfeicoamento do Pessoal de Nível Superior (CAPES). Centre National de la Recherche Scientifique pays the salary of C.B.-V.

Disclosures

None.

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that lack thrombospondin 2 display connective tissue abnormalities that are associated with disordered collagen fibrillogenesis, an increased vascular density, and a bleeding diathesis. *J Cell Biol.* 1998;140:419–430.


Thrombospondin-1 Is a Plasmatic Marker of Peripheral Arterial Disease That Modulates Endothelial Progenitor Cell Angiogenic Properties


Arterioscler Thromb Vasc Biol. 2011;31:551-559; originally published online December 9, 2010;
doi: 10.1161/ATVBAHA.110.220624

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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Thrombospondin-1 is a plasmatic marker of peripheral arterial disease that modulates endothelial progenitor cell angiogenic properties

David M. Smadja et al. Supplementary data

Study population
Consecutive PAD patients (n = 184) were enrolled in our vascular medicine department over a 2-years period. Patients were eligible if they were Caucasian men under 70 years of age with symptomatic atherosclerotic disease of the lower limbs and an ankle-brachial systolic pressure index (ABI) < 0.90, or a history of surgical or endovascular revascularization, as previously reported 1-3. They were excluded if they had non-atherosclerotic causes of PAD (cardioembolic disease, thromboangiitis obliterans, vasculitis, or congenital or metabolic vascular disease). Critical leg ischemia (CLI) was defined by pain at rest or non healing ulceration (> 2 weeks) plus an ankle systolic blood pressure below 50 mm Hg. Walking distance was defined as the maximum walking distance reported by the patient before stopping because of claudication. Smoking, hypertension and diabetes were significantly more frequent in the cases than in the controls. Cases were five times more likely to be on lipid-lowering drugs; as a result, they had lower total and LDL-cholesterol levels than the controls. Triglyceride levels were higher and HDL-cholesterol levels were lower in the cases. Interestingly, the cases had a lower mean body mass index (BMI), together with a lower hematocrit, a lower plasma protein concentration, and higher leukocyte and platelet counts. Renal function, based on serum creatinine levels, was similar in the two groups. Age-matched control subjects (n = 330) with no history of arterial disease (stroke, MI, angina, or PAD) were randomly selected among 703 Caucasian men composing a previously described control group used to study genetic risk factors for vascular thrombosis 4. They were recruited in a
healthcare center specializing in cardiovascular prevention, to which they had been referred for a routine check-up. All participants gave their written informed consent and the study protocol was approved by the Paris-HEGP -Broussais ethics committee.

**Blood collection**

Blood samples were collected in Vacutainer tubes (Becton-Dickinson Diagnostics, Le Pont-de-Claix, France) containing 0.105 M sodium citrate (1 vol/9 vol). Plasma was obtained by centrifugation at 2300 g for 10 minutes and was immediately stored at - 80°C until analysis.

**Local injection of bone marrow mononuclear cells (BM-MNC) in PAD patients with CLI**

The OPTIPEC clinical trial (Optimization of Progenitor Endothelial Cells in the Treatment of Critical Leg Ischemia; [clinicaltrials.gov](http://clinicaltrials.gov): NCT00377897) was a multicenter, phase I non-randomized study. The clinical findings have been published elsewhere 5. Briefly, patients were eligible for the protocol if they had CLI associated with limited gangrene or a non-healing ischemic ulcer and if they were not eligible for surgical revascularization or percutaneous angioplasty, or if such a procedure had little chance of success. The cell therapy protocol was similar to that initially published by Tateishi-Yuyama et al 6. Control histological sections were obtained from age- and sex-matched patients with critical limb ischemia who were not included in the cell therapy protocol and who were amputated during the same period.

**ELISA Assays**

Plasma levels of VEGF, PlGF and TSP-1 were measured with enzyme-linked immunosorbent kits from R&D Systems® (Minneapolis, MN, USA).
ECFC culture, *in vitro* angiogenesis assays, and *in vivo* Matrigel-plug assay

ECFC culture, *in vitro* angiogenesis assay and *in vivo* Matrigel-plug assay conditions have been described in detail elsewhere.\(^7\)\(^\text{-}^\text{12}\) \(^{TSP-1}\)\(^{-/-}\) mice, in which the *TSP-1* gene was disrupted by homologous recombination \(^13\), were kindly provided by Dr. A Bonnefoy \(^14\). For this study the *TSP-1* gene deficiency was bred into a C57Bl6/J background.

A1 peptide (TSP Hep I \(^15\)) derived from N-terminus of TSP-1 was synthesized in the Department of biophysics at UNIFESP ((Federal University of São Paulo, Escola Paulista de Medicina), using an automated bench-top simultaneous multiple solidphase peptide synthesizer (PSSM 8 System; Shimadzu, Tokyo, Japan).

ECFC transfection with siRNA against TSP-1

Short interfering RNA silencing *TSP-1* gene expression (sc-36665, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was mixed with the Primefect\(^\text{®}\) reagent (LONZA) at 10 \(\mu\)M to obtain transfection complexes, which were added to \(2\times10^5\) ECFC in complete EGM2 medium in 6-well plates. Control siRNA (Allstars Neg. control siRNA, Qiagen, Cambridge, MA, USA) was used in parallel.

Real-Time - quantitative Polymerase Chain Reaction (RT-qPCR)

The theoretical and practical aspects of RT-qPCR on the ABI Prism 7900 Sequence Detection System (Applied Biosystems) are described in detail elsewhere \(^10\). Rapidly, we quantified transcripts of the TBP gene, which encodes the TATA box-binding protein (a component of the DNA-binding protein complex TFIID) as the endogenous RNA control, and target gene expression was normalized on the basis of its TBP content. Primers for TBP and the target genes (sequences available on request) were chosen with the assistance of Oligo 5.0 software (National Biosciences, Plymouth, MN, USA).
**Hindlimb ischemia model.**

Nude mice underwent surgery to induce unilateral hindlimb ischemia as previously described and were then randomly allocated to intravenous injection of ECFC that had been transfected with control or TSP-1 siRNA. After 1 week, the ischemic/normal limb blood flow ratio was determined by using a laser Doppler perfusion imaging system (Moor instruments). C57Bl6/J mice underwent surgery to induce unilateral hindlimb ischemia left femoral artery and vein ligation and excision. Laser Doppler Perfusion Imaging was performed at day 14 in the C57Bl6/J mice Hindlimb ischemia model. Figure 3E: left panel: mice receiving 20 µL PBS per day by intra-muscular injections during 5 days; right panel: mice receiving 10 µg rhTSP-1 (R&D systems) per day, in 20 µL intra-muscular injections, during 5 days. Figure 3F: Perfusion ratio means for the C57Bl6/J mice Hindlimb ischemia model. n = 10 for each group, p = 0.04.

**Statistical analysis**

The nonparametric Mann-Whitney $U$ test was used to analyze differences between PAD patients and controls. Data from *in vitro* ECFC assays are reported as means ± SEM. Significant differences were identified by ANOVA followed by Fisher’s protected least-significant-difference test. StatView statistical software (Cary, NC 27513, USA) was used for all analyses, and p values below 0.05 were considered to denote significant differences.
Supplementary Figure I: ECFC express endothelial markers and TSP-1 receptor CD47 but not CD36
Supplementary Figure II: TSP-1 inhibition in cord blood derived ECFC

A. Quantitative analysis of TSP-1 mRNA by RT-qPCR, after 24, 48 and 72 hours of transfection with control-siRNA (All Star negative control, Qiagen®) and TSP-1-siRNA (Santa Cruz Biotechnologies®) using the Primefect® reagent kit (LONZA). The mean and SEM of three experiments are shown.*: p < 0.05

B. Quantitative analysis of TSP-1 protein by western blot, after 48 hours of transfection with control-siRNA (All Star negative control, Qiagen®) and TSP-1-siRNA (Santa Cruz Biotechnologies®) using the Primefect® reagent kit (LONZA).
Supplementary Figure III: Expression of CXCR4 on transfected cord blood-ECFC
Supplementary Figure IV: TSP-1 inhibition in adult blood derived ECFC and in HUVEC

A. Quantitative analysis of TSP-1 mRNA by RT-qPCR, after 24, 48 and 72 hours of transfection with control-siRNA (All Star negative control, Qiagen®) and TSP-1-siRNA (Santa Cruz Biotechnologies®) using the Primefect® reagent kit (LONZA). The mean and SEM of three experiments are shown. *: p < 0.05
B. Quantitative analysis of TSP-1 protein by western blot, after 48 hours of transfection with control-siRNA (All Star negative control, Qiagen®) and TSP-1-siRNA (Santa Cruz Biotechnologies®) using the Primefect® reagent kit (LONZA).

![Western Blot Image]

C. Quantitative analysis of SDF-1 and CXCR4 after transfection with control-siRNA (All Star negative control, Qiagen®) and TSP-1-siRNA (Santa Cruz Biotechnologies®) using the Primefect® reagent kit (LONZA) in adult blood derived ECFC and HUVEC.

![Graph Image]
Supplementary Figure V: effect of 1 and 10 µg/mL human recombinant TSP-1 on apoptosis related genes in cord blood derived ECFC.
Supplementary Figure VI: effect of 1 and 10 µg/mL human recombinant TSP-1 on cord blood derived ECFC.
Supplementary Figure VII: Effect of TSP-1 N-terminal peptide (10 μM) on cord blood derived ECFC differentiation in Matrigel in vitro.
Table 1. Characteristics of the Study Population

<table>
<thead>
<tr>
<th></th>
<th>Cases (n=184)</th>
<th>Control Subjects (n=330)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>57.1±7.2</td>
<td>56.7±7.6</td>
<td>0.6</td>
</tr>
<tr>
<td>Current/past smokers, %</td>
<td>97.8</td>
<td>47.0</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Hypertension, %</td>
<td>43.5</td>
<td>16.1</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Hypercholesterolemia, %</td>
<td>59.2</td>
<td>61.2</td>
<td>0.9</td>
</tr>
<tr>
<td>Lipid-lowering treatment, %</td>
<td>43.0</td>
<td>8.4</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Total cholesterol, mmol/L</td>
<td>4.97±1.23</td>
<td>6.10±0.93</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>LDL cholesterol, mmol/L</td>
<td>3.04±1.03</td>
<td>3.94±0.87</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/L</td>
<td>1.09±0.33</td>
<td>1.54±0.41</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
<td>1.96±1.36</td>
<td>1.41±0.87</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Diabetes, %</td>
<td>24.5</td>
<td>8.5</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Fasting blood glucose, mmol/L</td>
<td>6.5±2.4</td>
<td>5.9±0.7</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Asymptomatic, %</td>
<td>10.3</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>Intermittent claudication, %</td>
<td>75.6</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>Critical ischemia, %</td>
<td>14.1</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>Prior revascularization, %</td>
<td>55.4</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>Ankle-brachial systolic pressure index</td>
<td>0.64±0.14</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>Coronary heart disease, %</td>
<td>23.9</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>Cerebrovascular disease, %</td>
<td>10.4</td>
<td>...</td>
<td>...</td>
</tr>
</tbody>
</table>

Values are given as mean±SD or percentages. BMI indicates body mass index.
Table 2. Association of peripheral arterial disease with VEGF, PIGF, TSP-1 tertiles

<table>
<thead>
<tr>
<th></th>
<th>CASES</th>
<th>CONTROLS</th>
<th>P</th>
<th>OR (95%CI)</th>
<th>OR (95%CI)*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>VEGF</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;11.2</td>
<td>36.4 (67)</td>
<td>31.0 (77)</td>
<td>&lt;0.0001</td>
<td>2.62 (1.59-4.31)</td>
<td>4.11 (1.80-9.43)</td>
</tr>
<tr>
<td>11.2-18.0</td>
<td>19.6 (36)</td>
<td>44.0 (109)</td>
<td></td>
<td>1.00 (reference)</td>
<td>1.00 (reference)</td>
</tr>
<tr>
<td>&gt;18.0</td>
<td>44.0 (81)</td>
<td>25.0 (62)</td>
<td></td>
<td>3.95 (2.39-6.53)</td>
<td>2.28 (0.99-5.21)</td>
</tr>
<tr>
<td><strong>PIGF</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;6.7</td>
<td>39.1 (72)</td>
<td>29.9 (69)</td>
<td>0.005</td>
<td>2.09 (1.32-3.33)</td>
<td>5.03 (2.09-12.09)</td>
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<tr>
<td>6.7-9.8</td>
<td>24.5 (45)</td>
<td>38.9 (116)</td>
<td></td>
<td>1.00 (reference)</td>
<td>1.00 (reference)</td>
</tr>
<tr>
<td>&gt;9.8</td>
<td>36.4 (67)</td>
<td>31.2 (93)</td>
<td></td>
<td>1.84 (1.16-2.94)</td>
<td>0.84 (0.35-2.02)</td>
</tr>
<tr>
<td><strong>TSP-1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;215</td>
<td>9.1 (16)</td>
<td>50.4 (122)</td>
<td>&lt;0.0001</td>
<td>0.17 (0.09-0.32)</td>
<td>0.23 (0.09-0.60)</td>
</tr>
<tr>
<td>215-432</td>
<td>34.9 (61)</td>
<td>32.6 (79)</td>
<td></td>
<td>1.00 (reference)</td>
<td>1.00 (reference)</td>
</tr>
<tr>
<td>&gt;432</td>
<td>56.0 (98)</td>
<td>16.9 (41)</td>
<td></td>
<td>3.09 (1.88-5.07)</td>
<td>3.25 (1.40-7.08)</td>
</tr>
</tbody>
</table>

Odd ratios for the lowest and upper tertiles relative to the middle tertile were computed using unconditional logistic regression analysis adjusted on age.

* Additional adjustment for PAD risk factors (hypertension, hypercholesterolemia, diabetes and smoking status), lipid-lowering treatment and CRP.
Table 3. Association of PAD patient clinical characteristics with VEGF, PI GF, TSP-1 levels

<table>
<thead>
<tr>
<th></th>
<th>VEGF</th>
<th>PI GF</th>
<th>TSP-1</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Continuous factors</strong></td>
<td></td>
<td></td>
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<tr>
<td>Age</td>
<td>0.02</td>
<td>0.12 *</td>
<td>-0.07</td>
</tr>
<tr>
<td>BMI</td>
<td>0.01</td>
<td>0.08</td>
<td>-0.05</td>
</tr>
<tr>
<td>Glycaemia</td>
<td>-0.02</td>
<td>0.05</td>
<td>-0.003</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>-0.004</td>
<td>0.04</td>
<td>0.01</td>
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<tr>
<td>HDL cholesterol</td>
<td>-0.07</td>
<td>-0.03</td>
<td>0.07</td>
</tr>
<tr>
<td>LDL cholesterol</td>
<td>-0.004</td>
<td>0.04</td>
<td>-0.004</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>0.10</td>
<td>0.05</td>
<td>-0.004</td>
</tr>
<tr>
<td>CRP</td>
<td>0.16 *</td>
<td>0.24 *</td>
<td>0.001</td>
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<tr>
<td><strong>Categorical factors</strong></td>
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<tr>
<td>Diabetes</td>
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<tr>
<td>No</td>
<td>219 ± 7</td>
<td>238 ± 7</td>
<td>219 ± 6</td>
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<tr>
<td>Yes</td>
<td>214 ± 16</td>
<td>258 ± 17</td>
<td>219 ± 13</td>
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<tr>
<td>Current smoking</td>
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<tr>
<td>No</td>
<td>209 ± 8</td>
<td>237 ± 9</td>
<td>214 ± 7</td>
</tr>
<tr>
<td>Yes</td>
<td>232 ± 10</td>
<td>247 ± 11</td>
<td>227 ± 8</td>
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<tr>
<td>Hypertension</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>No</td>
<td>221 ± 8</td>
<td>225 ± 9 *</td>
<td>217 ± 7</td>
</tr>
<tr>
<td>Yes</td>
<td>214 ± 9</td>
<td>262 ± 10</td>
<td>223 ± 8</td>
</tr>
<tr>
<td>Hyperlipidemia</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>No</td>
<td>219 ± 9</td>
<td>246 ± 10</td>
<td>223 ± 8</td>
</tr>
<tr>
<td>Yes</td>
<td>217 ± 8</td>
<td>238 ± 8</td>
<td>217 ± 7</td>
</tr>
<tr>
<td>Lipid-lowering treatment</td>
<td></td>
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<tr>
<td>No</td>
<td>212 ± 7</td>
<td>242 ± 8</td>
<td>224 ± 6</td>
</tr>
<tr>
<td>Yes</td>
<td>234 ± 13</td>
<td>242 ± 14</td>
<td>208 ± 11</td>
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<tr>
<td>Antiagregants</td>
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<tr>
<td>No</td>
<td>206 ± 9</td>
<td>256 ± 10 *</td>
<td>226 ± 8</td>
</tr>
<tr>
<td>Yes</td>
<td>233 ± 11</td>
<td>220 ± 12</td>
<td>212 ± 9</td>
</tr>
</tbody>
</table>

* p < 0.05 (Partial spearman correlation analysis or nonparametric analysis of variance adjusted on case-control status). Partial Spearman correlation or adjusted mean (±SEM) ranks are shown after pooled PAD cases and controls together.
Table 4. Association of severity of peripheral arterial disease with VEGF, PI GF, TSP-1 levels

<table>
<thead>
<tr>
<th>Grade of Severity</th>
<th>1, n=102</th>
<th>2, n=44</th>
<th>3, n=38</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF</td>
<td>17.1 (9.7-25.9)</td>
<td>12.9 (7.0-25.6)</td>
<td>15.0 (6.4-30.8)</td>
<td>0.43</td>
</tr>
<tr>
<td>PI GF</td>
<td>7.1 (5.1-10.6)</td>
<td>9.3 (5.3-12.1)</td>
<td>8.7 (6.9-13.4)</td>
<td>0.07</td>
</tr>
<tr>
<td>TSP-1</td>
<td>430 (308-588)</td>
<td>468 (345-584)</td>
<td>513 (379-628)</td>
<td>0.59</td>
</tr>
</tbody>
</table>

Median (interquartile range) are shown and compared using nonparametric analysis of variance.

We classified cases according to clinical criteria of PAD severity (Fontaine-Leriche stages, WHO classification): grade 1 = intermittent claudication with maximal walking distance > 100 meters; grade 2 = intermittent claudication with maximal walking distance < 100 meters; grade 3 = critical leg ischemia.


