Thrombospondin-4 and Its Variants
Expression and Differential Effects on Endothelial Cells

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Background—In a recent large-scale genetic association study, a single nucleotide polymorphism in the thrombospondin-4 (TSP-4) gene, resulting in a proline-for-alanine substitution at position 387, was associated with a significantly increased risk for premature atherosclerosis. TSP-4 had not previously been implicated in vascular pathology, and very little information is available on its expression and functions.

Methods and Results—The goal of this study was to assess TSP-4 expression in vessel wall and to identify differences in functions of TSP-4 variants that could account for the proatherogenic effects of the (P387)TSP-4 variant. TSP-4 expression was demonstrated in human endothelial cells (ECs) and vascular smooth muscle cells from brain blood vessels and coronary arteries. (P387)TSP-4 and its fragment (residues 326 to 722), but not the A(387) forms, suppressed EC adhesion and proliferation. The (P387)TSP-4 was more active in inducing the phosphorylation of focal adhesion kinase, consistent with inhibition of proliferation. Both variant fragments increased the proliferation of human aortic smooth muscle cells.

Conclusions—TSP-4 is expressed by vascular cells and influences the vessel wall by modulating the proliferation of ECs and smooth muscle cells. The A387P substitution is a “gain-of-function” mutation, favoring a form of TSP-4 that interferes with EC adhesion and proliferation and may thereby be proatherogenic. (Circulation. 2003;108:1514-1519.)

Key Words: cells, adhesion • cells, proliferation • atherosclerosis • extracellular matrix • endothelium

The search for genes involved in atherogenesis has been greatly accelerated by the development of high-throughput strategies. As specific genes are implicated in atherogenesis, the next major challenge is to determine how their gene products influence development of vascular lesions. Such information concerning risk factor genes is essential to understand the mechanisms underlying atherogenesis and ultimately to use the gene association data for treatment and prevention of cardiovascular disease.

In one of the large-scale genetic association studies that has been undertaken to identify genes involved in premature atherosclerosis, an impressive statistical association was found with 3 single nucleotide polymorphisms (SNPs), all members of the thrombospondin (TSP) family.1 The clustering of these “risk” genes within the TSP family assigns a previously unanticipated pathophysiological importance to these matrix proteins. In the TSP-4 gene, a proline rather than an alanine at residue 387 had a strong association with myocardial infarction (odds ratio, 1.89) and was expressed at high frequency. The association of (P387)TSP-4 with premature atherosclerosis has been confirmed by an independent study in a distinct patient population.2 Very little information is available on TSP-4 or its functions. Its mRNA expression is limited to heart, brain, and skin of adults,3 but the cell types producing mRNA or its translation into protein have not been demonstrated.

In view of the recent association of TSP-4 with atherogenesis and yet the dearth of information on its expression, we sought to determine whether TSP-4 is expressed by vascular cells. In addition, TSP-4 variants and variant fragments containing portions of the region surrounding the SNP were expressed, and their differential effects on key vascular cell responses, adhesion and proliferation, were assessed.

Methods

Expression and Purification of TSP-4 Variants
TSP-4 cDNA was obtained from Dr Lawler. The mutation was introduced by site-directed mutagenesis using the QuickChange Site-Directed Mutagenesis Kit (Stratagene). cDNAs were cloned into pcDNA3 vector, and TSP-4 variants were stably expressed in HEK293 cells. TSP-4 variants were purified as described.4

Expression and Purification of TSP-4 Fragments
TSP-4 variant fragments were expressed in SMCs from human aortas (HASMCs) as secreted GST fusion proteins using pGLEX vector obtained from Dr W.E. Fahl (University of Wisconsin, Madison)5 and the TSP-4 signal peptide sequence upstream of GST. cDNA of the TSP-4 fragment, residues 326 to 722, which includes type 2 and type 3 repeats,3 was obtained by reverse transcription–polymerase chain reaction (RT-PCR)
from human brain RNA (Clontech) using primers 5'-GCAGCGCCGGTCTAGTCTGTTGCACGAGT and 5'-GCAGCGCCGGTGTTGGATGTCGAAATACCACCCTCACC and cloned downstream of the GST cDNA. The resulting cDNA produced the secreted fusion protein having GST on the N-terminus of the TSP-4 fragment. The TSP-4 variant fragments were purified on anti-GST resin prepared with polyclonal anti-GST antibody (Amersham).

**RNA Isolation and RT-PCR**

RNA was isolated using Trizol reagent (Invitrogen). RT-PCR was performed using reagents from Roche Laboratories. To detect mRNA of TSP-4 in different cell types, a sequence-specific primer, 5'-TGTATAAGCCTAGCAGGAAAATGG-3', was used for the RT reaction, and primers 5'-GCGGCCGACAGTGGATGTCGAAATACCACCCTCACC and 5'-TGTATAAGCCTAGCAGGAAAATGG-3' were used for PCR amplification of the 400-bp fragment.

**Transfection of HEK293 Cells, HASMCs, and Human Umbilical Vein Endothelial Cells**

HEK293 cells and HASMCs were transfected by use of Lipofectamine Plus reagent (Invitrogen) (9 μg/100-mm plate). Fifteen individual clones of HEK293 cells and HASMCs for each transfected plasmid were analyzed. Human umbilical vein endothelial cells (HUVECs) were transfected with ExGen500 Reagent (ME Biotech). The efficiency of transient transfection (~15%) was monitored by use of the enhanced green fluorescent protein vector from Clontech. TSP-4 variants and variant fragments were secreted and detected in media in equal amounts by ELISA.

**Immunostaining of Brain Tissue**

Free-floating 30-μm sections of human brain (HB) from patients undergoing surgery for epilepsy were incubated overnight at 4°C with anti–human TSP-4 antibody (Santa Cruz) and for 3 hours at room temperature with secondary anti–goat IgG fluorescein-conjugated antibody (Jackson Laboratories). Sections were mounted with Vectashield mounting medium (Vector Laboratories).

**Immunoprecipitation of TSP-4 From the Supernatants of Cultured HBECs**

[^35]Methionine (Amersham) (100 μCi/75 cm², 6 mL of growth medium) was added to the cells for 24 hours. Conditioned media were incubated overnight at 4°C with anti–TSP-4 antibody (4 μg/sample). The next day, rec-protein G–Sepharose conjugate (ZYMED) (100 μL/sample) was added for 4 hours. The protein was eluted in 4X SDS-PAGE sample buffer.

**Isolation and Culture of HBECs and HBSMCs**

ECs were isolated from secondary branches of middle cerebral arteries of patients undergoing temporal lobectomies as described. After EC dissociation, the remaining vessel tissue was allowed to attach for 20 minutes and covered with DMEM/F-12 (1:1), 10% FBS. SMCs migrating out of the tissue were harvested. The purity of the EC and SMC populations was confirmed by immunostaining for cell surface markers.

**Adhesion Assays**

Twenty-four-well plates were incubated overnight at 4°C with purified TSP-4 variants (20 μg/well), variant fragments, or conditioned media from 6 HASMC clones expressing recombinant TSP-4 fragments. For conditioned media, the amounts of media per well were adjusted to produce equal GST activity in the quantitative GST activity assays. The next day, 50 000 cells/well were plated and incubated 30 minutes at 37°C. Cells were washed with PBS, and the amount of DNA/well was measured with the CyQuant Cell Proliferation Assay kit (Molecular Probes).

**Proliferation Assays**

Cell proliferation was measured as described for adhesion assays. A total of 20 000 HASMCs/well or 50 000 HUVECs/well were plated. A total of 20 000 HASMCs/well or 50 000 HUVECs/well were plated. Twenty-four-well plates were incubated overnight at 4°C with purified TSP-4 variants (20 μg/well), variant fragments, or conditioned media from 6 HASMC clones expressing recombinant TSP-4 fragments. For conditioned media, the amounts of media per well were adjusted to produce equal GST activity in the quantitative GST activity assays. The next day, 50 000 cells/well were plated and incubated 30 minutes at 37°C. Cells were washed with PBS, and the amount of DNA/well was measured with the CyQuant Cell Proliferation Assay kit (Molecular Probes).

**Results**

**Expression of TSP-4 in Vascular Cells**

To determine whether vessel wall cells can express TSP-4, RNA of cultured ECs and SMCs from brain (HBECs and HBSMCs) and coronary artery (HCAECs and HCSMCs) were subjected to RT-PCR. As shown in Figure 1A and B, TSP-4 mRNA was detected in multiple cell lines. To confirm that TSP-4 mRNA is translated, we examined TSP-4 protein expression by ECs. TSP-4 protein was detected in brain tissues by immunofluorescence in capillaries (Figure 1C). The sensitivity of the assay was 125 ng free GST. Amounts of fusion proteins per well of 6-well or 24-well plates corresponded to 385 ng or 80 ng free GST, respectively.

**Statistics**

Probability values were obtained with a t test (Microsoft Excel Program) for mean values from ≥3 experiments performed in quadruplicate.
Although mRNA of TSP-4 was detected by RT-PCR in coronary artery cells, immunoprecipitation from the conditioned media or lysates of these cells under the same conditions as for the HBECs did not yield TSP-4 protein.

**Expression of Recombinant TSP-4 Variants in HEK293 Cells**

TSP-4 variants were stably expressed in HEK293 cells. Although transfection efficiencies were similar, we were unable to propagate HEK293 cells expressing (P387)TSP-4 or its fragment, presumably because of its de-adhesive properties (see below). Hence, HEK293 cells expressing (P387)TSP-4 were always provided an adhesive substrate; they were grown on poly-lysine. Purified variants had identical electrophoretic mobility in both reducing and nonreducing SDS-PAGE, suggesting similar processing, including oligomerization (Figure 3, A and B). Similar amounts of (P387)TSP-4 and (A387)TSP-4 bound to the cell culture plastic, as determined by bicinchoninic assay and anti–TSP-4 ELISA after 24 hours of incubation at 4°C (data not shown).

**Expression of Recombinant TSP-4 Variant Fragments in HASMCs**

All TSPs are composed of domains, which fold and function independently in many of the interactions with cell-surface receptors and other matrix proteins.8–12 Thus, fragments are often used to dissect the complex functions of the TSPs and to analyze activities that manifest on unfolding and/or degradation of the TSPs.13,14 Accordingly, we assessed the differential functions of TSP-4 variant fragments of ≈400 amino acids, which harbor residue 387 and consist of 3 type 2 and 7 type 3 TSP repeats. This region of TSP-4 also includes an RGD integrin recognition site in its third Ca²⁺-binding repeat, providing a rationale for examining the effects of these fragments on cell adhesion and proliferation. The fragments were stably expressed as secreted GST fusion proteins in HASMCs. HASMCs expressed the variant TSP-4 fragments in a similar fashion, both in terms of the number of expressing clones and the amount of secreted proteins. Six high-expressing clones in each group were selected for subsequent experiments. Fragments purified on anti-GST resin had similar size by SDS-PAGE (Figure 3C). However, we were unable to purify sufficient amounts of fragments using glutathione-Sepharose, probably because of compromised GST activity, and fragments purified on anti-GST resin had reduced activity in adhesion and proliferation assays, probably because of the low-pH conditions used for the elution. Therefore, in some experiments with fragments, the conditioned medium from SMCs stably expressing the fusion

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**Figure 2.** Expression of TSP-4 protein by vascular cells in human brain. A, Sections of human brain were stained with anti–TSP-4 antibody followed by anti-goat fluorescein-conjugated antibody. Anti–von Willebrand factor (vWF) antibody was used as an EC marker to confirm localization of TSP-4 to blood vessels (arrows). B, TSP-4 was immunoprecipitated from supernatants of individual HBEC cell lines after metabolic labeling with [³⁵S]methionine and analyzed by SDS-PAGE on a 10% gel under reducing conditions. Cell lines 1 and 2 expressed (A387)TSP-4; cell line 3 expressed (P387)TSP-4.

**Figure 3.** Expression and purification of recombinant TSP-4 variants and variant fragments. A, SDS-PAGE (6%) of purified TSP-4 variants: left, nonreduced (pentamers); right, reduced (monomers); Coomassie blue. A, (A387)TSP-4; P, (P387)TSP-4. B, Western blotting of same samples with anti–TSP-4 antibody. C, TSP-4 variant fragments of predicted size ≈74 kDa purified on anti-GST resin; Coomassie blue.
proteins was used. To obtain evidence that the effect of conditioned media is due to the presence of P387 TSP-4 fragment, media depleted of TSP-4 fragments on glutathione resin were used as a control.

**Effect of (P387)TSP-4 on the Adhesion of HUVECs**

Wells of tissue culture plates were coated with purified TSP-4 variants (20 μg/well overnight at 4°C), and HUVECs were incubated in the wells for 30 minutes at 37°C. After washing, the adherent cells were quantified as the amount of DNA per well. There was a significant decrease in amounts of DNA/well both with full-length (P387)TSP-4 and with its fragment: intensity of fluorescence was 92±37 relative fluorescence units (RFU) with (P387)TSP-4 versus 251±15 RFU with (A387)TSP-4 (P=0.003) and 1118±42 RFU with the (P387) fragment versus 1585±94 RFU with the (A387) fragment (P=0.002) (Figure 4, A and B).

When tissue-culture plastic was coated with conditioned medium from HASMCs expressing TSP-4 fragments or with the same medium depleted of the TSP-4 fragments by preincubation with glutathione-Sepharose, adhesion of HUVECs was decreased by the (P387)TSP-4 fragment but not by the (A387)TSP-4 fragment relative to the GST control (P<0.05). This effect of the (P387)TSP-4 fragment was abolished by depletion of conditioned media with glutathione-Sepharose (Figure 4C). Thus, the TSP-4 variants and their fragments differed in their capacity to support cell adhesion.

**Effect of (P387)TSP-4 on the Proliferation of HUVECs**

During the first hours of culturing HUVECs in the presence of TSP-4 variants, there was little difference in the appearance of cells adhering to (A387)TSP-4 and (P387)TSP-4, although fewer cells attached and spread on (P387)TSP-4. However, after 24 hours, morphological differences became striking: on (A387)TSP-4, HUVECs spread, and the cultures reached confluence after several days; but on (P387)TSP-4, the attached HUVECs failed to spread and most detached from the surface (Figure 4D). There was no significant proliferation in the presence of (P387)TSP-4: DNA fluorescence on day 4 was 83.5±6.6 RFU, compared with 1813.5±87 RFU on day 4 in the presence of (A387)TSP-4 (P=0.017) (Figure 5A). When HUVECs were transiently transfected with the TSP-4 variants, the same antiproliferative effect of (P387)TSP-4 was observed. However, this effect was delayed (Figure 5B), probably reflecting the time for the transfected cells (~15% of the total cell number) to produce sufficient protein to affect the majority of cells in the cultures.

There was a significant difference in cell number at 24 hours of culturing: 342±32 RFU for (P387)TSP-4 versus 537±20 RFU for (A387)TSP-4 (P=0.01); and the increase decreased dramatically over time (day 6, 307±23 RFU versus 1259.5±69 RFU, respectively, P=0.0002). The cultures of HUVECs plated in wells coated with conditioned media containing the fragments or GST were maintained for 5 days to assess proliferation (Figure 6A). Cell number was the same for cells maintained in the presence of GST or (A387)TSP-4 fragment, but cell number was reduced by 34% (P<0.05) in the presence of (P387)TSP-4 fragment. When ECs were transiently transfected with variant TSP-4 fragments or GST

![Figure 4](image-url)  
**Figure 4.** Effect of TSP-4 variants and their fragments on adhesion of HUVECs. Cells were plated on plastic preincubated with purified TSP-4 variants (A), purified variant fragments (B), or conditioned (cond.) media from HASMCs expressing TSP-4 variant fragments (C). A, B and C, Cells (50 000 cells/well of 24-well plate) were incubated 30 minutes at 37°C and washed with PBS, and amount of DNA/well was measured. D, Cells were plated on plastic preincubated with purified TSP-4 variants (50 000 cells/well of 24-well plate), and photographs were taken after 24 hours of culture: left, (A387)TSP-4; right, (P387)TSP-4.

![Figure 5](image-url)  
**Figure 5.** Effect of (P387)TSP-4 on proliferation of ECs. A, HUVECs (50 000 cells/well of 24-well plate) were grown on plastic preincubated with purified TSP-4 variants, and amount of DNA/well was measured. B, HUVECs (50 000 cell/well) transiently expressing TSP-4 variants were cultured for 1 to 6 days, and amount of DNA/well was measured.
alone, the number of cells, quantified on the basis of cellular DNA, also was consistently lower 72 to 96 hours after transfection with (P387)TSP-4 fragment compared with (A387)TSP-4 fragment or GST alone (Figure 6B). DNA fluorescence was 585\pm134 RFU with (P387)TSP-4 fragment compared with 960\pm150 RFU with (A387)TSP-4 fragment or 1020\pm230 RFU in control, P<0.05. Nevertheless, there was no change in apoptosis as assessed by caspase 3 activity or in cell cycle progression as assessed by propidium iodide staining by fluorescence-activated cell sorting 48 to 96 hours after transfection between the cells expressing (A387)TSP-4 fragment, (P387)TSP-4 fragment, or GST alone (not shown).

**Effect of (P387)TSP-4 and Its Fragment on FAK Phosphorylation in ECs**

Cell adhesion activates intracellular kinases, including FAK. FAK phosphorylation occurs during the initial stages of cell adhesion and then declines as the cells form focal adhesion complexes and reorganize their actin cytoskeleton.15,16 Lysates from HUVECs transiently expressing TSP-4 were prepared 48 hours after transfection, and phosphoFAK was detected by Western blotting. FAK phosphorylation was increased 2.7-fold in cells expressing (P387)TSP-4 compared with cells expressing (A387)TSP-4 or 3.7-fold compared with cells transfected with control plasmid (Figure 7A). In addition, HUVEC lysates were prepared 30 and 60 minutes after plating onto wells coated with the 2 variant TSP-4 or their fragments, and phosphoFAK was detected by Western blotting (Figure 7, B and C). In the presence of (P387)TSP-4 or its fragment, FAK phosphorylation was more extensive and endured longer than with (A387)TSP-4 and the control. Densitometric scans of the gels indicated that the phosphorylation was increased 2.2-fold in the presence of (P387)TSP-4 and 1.5-fold in the presence of the (P387)TSP-4 fragment compared with the (A387)TSP-4 variant and 1.5-fold and 4.2-fold compared with control cells. FAK phosphorylation was still greater with the (P387)TSP-4 fragment at 60 minutes (1.8-fold), but an effect with (A387)TSP-4 fragment was no longer detected.

**Effects of the TSP-4 Fragments on Adhesion and Proliferation of HASMCs**

Under the same conditions as used to assess HUVEC adhesion, the variant TSP-4 fragments did not differentially affect HASMC adhesion (not shown). In addition, we examined the proliferation of HASMC clones expressing the variant TSP-4 fragments or GST alone. As shown in Figure 8, the clones expressing the TSP-4 fragments increased proliferation compared with control clones expressing GST alone, but there was no difference in the effects of the (A387)TSP-4 fragment and (P387)TSP-4 fragment.

**Discussion**

Previous reports on TSP-4 expression had shown that its mRNA is limited to heart, brain, and skin of adults. Our results show that TSP-4 mRNA is expressed by ECs and SMCs derived from brain blood vessels and coronary arteries. Cultured brain ECs secreted TSP-4 protein, and the protein was detected...
in brain capillaries by immunofluorescence in situ. The expression of TSP-4 protein and mRNA by brain ECs and SMCs suggests that the observed functional activities of the TSP-4 fragments may be exerted in vivo. Although cultured HCAECs and HCASMCs did not express detectable TSP-4 protein, mRNA was detected in both cell types, suggesting that the protein may be rapidly produced and secreted in coronary arteries. Brain ECs appeared to produce and secrete both TSP-4 variants, suggesting that the proatherogenic influences arise from functional rather than expression differences.

We focused our studies on vascular cells, which would be exposed to secreted TSP-4. We found a differential effect of the variants on ECs: the (P387)TSP-4 and its fragment decreased EC adhesion. FAK phosphorylation was increased by the (P387)TSP-4 and its fragment, suggesting that the decreased adhesion may arise from an inhibition of the transition from initial to strong adhesion, which is characterized by dephosphorylation of FAK, formation of focal adhesion contacts, and actin cytoskeletal rearrangements. The inhibition of this transition would favor a reversible adhesive state and could lead to de-adhesion.\(^1\) In addition, compared with the (A387)TSP-4 and its fragment, (P387)TSP-4 and its fragment suppressed EC proliferation. This effect on proliferation may be a consequence of the differences in cell adhesion in presence of the (P387)TSP-4 and its fragment. Changes in cell adhesion, including cytoskeletal rearrangements, are tightly linked to integrin engagement, which can consequently alter cell proliferation.\(^1\) The identity of the integrin(s) or other receptors involved in TSP-4 recognition is a particularly important avenue for future analyses in view of the absence of the defined integrin recognition sites found in TSP-1,\(^1\)\(^\text{other than RGD, in the TSP-4 molecule. In vascular cells, failure to adhere or to undergo the transition to the strong adhesion results in inability to proliferate.}\(^1\) Together, these effects of the (P387)TSP-4 on EC adhesion and proliferation would alter the capacity of ECs in the vessel wall to respond to injury, creating a milieu for development of atherosclerotic lesions. It is noteworthy that the effects of (P387)TSP-4 are exerted in the presence of other TSPs (TSP-1 and TSP-2), which are produced and secreted by our cultured ECs. This suggests a unique or a dominant effect of the (P387)TSP-4 variant.

TSP-1 is a strong stimulator of SMC proliferation,\(^1\)\(^\text{–}\)\(^2\) and this signal depends on its midsegment.\(^2\) SMC clones expressing TSP-4 fragments proliferated faster than control clones expressing only GST. Thus, the pro-proliferative effect on SMCs may be a conserved function of the midsegments of the TSPs. However, this effect was not different in clones expressing (P387)TSP-4 or (A387)TSP-4 fragments.

The results of this study provide the first evidence that TSP-4 can regulate proliferation of vascular ECs and SMCs and that the mutation A387P can affect the activity of TSP-4 in a proatherogenic manner. TSP-4 is expressed by ECs and SMCs, suggesting that the (P387)TSP-4 may affect the cellular dynamics of the vessel wall. The proatherogenic variant P387 exerts a “gain-of-function” activity, interfering with EC adhesion and proliferation. This gain in function was observed with the (P387)TSP-4 fragment and intact (P387)TSP-4, suggesting that local conformational differences between the fragments are retained or accentuated in the pentameric TSP-4 molecules. These inhibitory effects of the (P387)TSP-4 on EC repair functions, coupled with its stimulatory effects on SMCs, may predispose the vasculature to the initiation and development of atherosclerotic lesions.

Acknowledgments

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