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Clinical Research

Thrombospondin 1 Is Increased in the Aorta and Plasma of Patients With Acute Aortic Dissection

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ABSTRACT

Background: Previous studies have shown that thrombospondin 1 (TSP-1) is involved in cardiovascular diseases, such as atherosclerosis and abdominal aortic aneurysm. However, TSP-1 expression levels in human aortic dissection (AD) remain unknown.

Methods: TSP-1 levels were detected in aortas collected from control subjects and AD patients. The TSP-1, interleukin (IL) 6, matrix metalloproteinase (MMP) 2, and MMP9 levels in plasma from non-AD patients and AD patients were measured. In addition, the effects of recombinant mouse TSP-1 protein on macrophage differentiation and smooth muscle cell (SMC) apoptosis were investigated.

Results: Compared with the aortas from control subjects, aortas from AD patients showed a significant increase in TSP-1 expression, especially in the torn sections. SMCs and endothelial cells produced TSP-1, but SMCs were the main source. TSP-1, IL-6, MMP2, and MMP9 levels were higher in AD patients than in non-AD patients, and plasma IL-6, MMP2, and MMP9 levels were positively correlated with TSP-1 levels

RÉSUMÉ

Contexte : Des études antérieures ont montré que la thrombospondine-1 (TSP-1) joue un rôle dans les maladies cardiovasculaires comme l'athérosclérose et l'anévrisme de l'aorte abdominale. Toutefois, les taux d'expression de la TSP-1 chez les patients présentant une dissection aortique (DA) demeurent inconnus.

Méthodologie : Les concentrations de TSP-1 ont été mesurées dans des aortes prélevées chez des sujets témoins et des patients présentant une DA. Les concentrations plasmatiques de TSP-1, d'interleukine-6 (IL-6) et de métalloprotéase matricielle-2 et -9 (MMP-2 et MMP-9) ont été mesurées chez des patients atteints de DA et chez des patients non atteints. De plus, nous avons étudié les effets de la protéine TSP-1 murine recombinante (rmTSP-1) sur la différenciation des macrophages et sur l'apoptose des cellules des muscles lisses (CML).

Résultats : Comparativement à celles des sujets témoins, les aortes des patients ayant une DA présentaient une augmentation marquée

Aortic dissection (AD) is a severe clinical vascular disease that is characterized by injury to the aortic wall. The incidence of AD is approximately 2.6-3.6 cases per 100,000 per year, and AD can lead to serious complications such as hemorrhagic shock, acute kidney failure, and even sudden death.[1] One previous study reported that the likelihood of fatality within 1 hour is as high as 1% to 2% in untreated patients.[2] Although the specific mechanisms of AD remain unknown, many studies have indicated that the inflammatory response is closely related to the onset and progression of AD.[3]

Thrombospondin 1 (TSP-1) is a 450-kDa extracellular matrix (ECM) glycoprotein, which acts as an endogenous inhibitor in angiogenesis and regulates cell proliferation.[4] TSP-1 was observed to exhibit anti-inflammatory and proinflammatory effects, and its specific effects seem to be closely related to the inflammatory microenvironment. A previous study reported that TSP-1 could promote the release of macrophage-derived interleukin (IL)-10 and play a protective role in lung injuries.[5] Deletion of TSP-1 resulted in more severe inflammation and aggravated cutaneous hypersensitivity reactions in mice.[6] In another study, TSP-1 was reported to have a proinflammatory effect and further exacerbate pulmonary hypertension.[7]

A considerable amount of evidence suggests that TSP-1 is involved in smooth muscle cell (SMC) apoptosis, endothelial

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in AD patients. Simple linear regression analysis and multivariate linear regression analysis showed that TSP-1 levels were independently correlated with the onset of AD. In cultured cells, recombinant mouse TSP-1 further increased inducible nitric oxide synthase (iNOS) mRNA expression in angiotensin (Ang) II-treated macrophages, whereas it reduced B-cell lymphoma-2 (Bcl2) mRNA levels and increased Bcl2-associated X protein (Bax) mRNA levels in Ang II-treated SMCs.

Conclusions: TSP-1 level is significantly increased in AD patients and might participate in AD via promoting classically activated macrophage (M1) macrophage differentiation and SMC apoptosis.

de l'expression de la TSP-1, en particulier dans les sections déchirées. Les CML et les cellules endothéliales produisaient de la TSP-1, mais les CML en constituaient la source principale. Les concentrations de TSP-1, d'IL-6, de MMP-2 et de MMP-9 étaient plus élevées chez les patients atteints de DA que chez les patients non atteints, et une corrélation positive entre les taux plasmatiques d'IL-6, de MMP-2 et de MMP-9 et ceux de TSP-1 a été observée chez les patients atteints de DA. Les analyses de régression linéaire simple et multivariée ont montré que les concentrations de TSP-1 étaient indépendamment corrélées à la survenue d'une DA. Dans des cellules en culture, la TSP-1 de souris recombinante a de plus provoqué une augmentation de l'expression de l'ARNm de l'oxyde nitrique synthase inductible (iNOS) dans les macrophages traités par l'angiotensine II, alors qu'elle réduisait les concentrations d'ARNm de la protéine Bcl-2 et augmentait les concentrations d'ARNm de la protéine Bax dans les CML traitées par l'angiotensine II.

Conclusions : La TSP-1 est présente à des concentrations significativement plus élevées chez les patients atteints de DA et pourrait participer à la DA en stimulant la différenciation des macrophages M1 et l'apoptose des CML.

cell senescence, and angiogenesis inhibition, which are closely related to the onset of cardiovascular diseases.[8-12] Furthermore, data from clinical experiments and animal studies showed that TSP-1 participates in hemorrhagic stroke, abdominal aortic aneurysm, and coronary atherosclerosis disease via the mechanisms described previously.[13-15] Because the expression of TSP-1 in human AD remains unknown, this study aimed to detect TSP-1 expression in human AD and explore a possible mechanistic link.

Methods

Collection of aorta samples and peripheral blood samples

Normal aortic samples (control group, n = 10) were obtained from heart donors who were declared brain-dead and had no significant cardiovascular diseases, and the aortic tissues showed no signs of pathology. The torn aortas (AD group, n = 12) were collected from patients who suffered from acute AD and received surgical treatment. Moreover, consecutive patients (n = 133) who suffered from acute chest pain and were hospitalized at the People's Hospital of Guangxi Zhuang Autonomous Region from March 2016 to August 2017 were enrolled in the study. After computed tomographic angiography and return to the intensive care unit, blood samples were collected. Some of these patients were excluded because of a history of cancer (n = 2), coronary artery disease (n = 7), peripheral arterial disease (n = 2), valvular heart disease (n = 3), or primary pulmonary hypertension (n = 2). The remaining patients were divided into a non-AD (NAD) group (n = 31) and an AD group (n = 86) according to the clinical symptoms and results of the computed tomography angiography. The AD group was further divided into Stanford A (n = 44) and Stanford B groups (n = 42). Plasma was collected and stored at −80°C for later experiments after the blood samples were centrifuged at 4000*g* for 20 minutes. Patients or their families provided informed consent. This study protocol was approved by the Medical Ethics Committee of the People's Hospital of Guangxi Zhuang Autonomous Region.

Western blot analysis

Aorta samples were lysed, the homogenate was collected, and the concentration of total protein in each sample was quantified. Approximately 20 μg protein from each sample was separated using sodium dodecyl sulphate-polyacrylamide gel electrophoresis, and then the proteins were transferred to polyvinylidene fluoride membranes (Millipore, Burlington, MA). After blocking in nonfat milk for 1 hour, the polyvinylidene fluoride membranes were incubated with anti−TSP-1 antibody and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody at 4°C overnight. After further incubation with a secondary antibody for 1 hour at room temperature, the blots were scanned using a 2-colour infrared imaging system (Odyssey; LI-COR Biosciences, Lincoln, NE).

Immunofluorescence

The aortic tissues were immersed in 4% neutral paraformaldehyde, embedded in paraffin, and cut into 4- to 5-μm sections. Immunofluorescent staining was used to detect TSP-1 expression in each sample. Double immunofluorescent staining with combinations of anti-CD68 antibody and anti−TSP-1 antibody, anti-CD3 antibody and anti-TSP-1 antibody, anti-CD31 antibody and anti−TSP-1 antibody, and anti−alpha smooth muscle action antibody and anti−TSP-1 antibody was performed to label the source of TSP-1 in macrophages, T lymphocytes, endothelial cells, and SMCs.

Enzyme-linked immunosorbent assay

All plasma samples were removed from the −80°C environment and defrosted at room temperature. Then, the TSP-1 (R&D Systems, Minneapolis, MN), IL-6, matrix metalloproteinase (MMP)-2 and MMP9 (eBioscience, San Diego, CA) levels in each sample were measured using enzyme-linked immunosorbent assay kits following the manufacturer's instructions.

Table 1. Quantitative polymerase chain reaction primers used in the study

Gene	Forward primer	Reverse primer
iNOS	TGACGCTCGGAACTGTAGCA	CAGTGATGGCCGACCTGAT
IL-6	AGTTGCCTTCTTGGGACTGA	TCCACGATTTCCCAGAGAAC
MMP2	AGATCTTCTTCTTCAAGGACCGGTT	GGCTGGTCAGTGGCTTGGGGTA
MMP9	TGGTGTGCCTGGAACTCA	GGAAACTCACACGCCAGAAGA
Bax	CTGAGCTGACCTTGGAGC	GACTCCAGCCACAAAGATG
Bcl2	GACAGAAGATCATGCCGTCC	GGTACCAATGGCACTTCAAG
GAPDH	AACTTTGGCATTGTGGAAGG	CACATTGGGGGTAGGAACAC

Cell isolation and culture

Bone marrow-derived macrophages (BMDMs) were isolated from C57BL/6 mice (8-9 weeks old) as previously described.[16,17] Briefly, mice were anaesthetized with 2% isoflurane and euthanized, then the tibias and femurs were isolated. The bone marrow cells were obtained by flushing the tibias and femurs. Complete Dulbecco's minimum essential medium (DMEM) (10% fetal calf serum, 50 U/mL penicillin, 50 g/mL streptomycin) with murine macrophage colony-stimulating factor (50 ng/mL) was used for the isolation, culture, and stimulation of the macrophages. The cells were cultured for 5 days to allow macrophage differentiation. Spleens were isolated from wild type mice, and a single cell suspension was prepared. CD4 positive T lymphocytes (CD4 TCs) were positively selected using CD4 magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) and an autoMACS separator. The CD4 TCs were cultured in RPMI 1640 complete culture medium and activated by treatment with anti-CD3 antibody and anti-CD28 antibody. The BMDMs and CD4 TCs were divided into 4 groups as follows: (1) BMDMs; (2) BMDMs and angiotensin (Ang) II; (3) BMDMs and CD4 TCs and Ang II; and (4) BMDMs and CD4 TCs and Ang II and recombinant mouse TSP-1 protein (rmTSP-1). Cells from all 4 groups were plated in complete DMEM (10% fetal calf serum, 50 U/mL penicillin, 50 μg/mL streptomycin) at densities of 5×10^5 and 2.5×10^6 for BMDMs and CD4 TCs, respectively. Moreover, the concentrations of Ang II and rmTSP-1 used were 100 nmol/L and 0.1 μmol/L, respectively. After culturing for 24 hours, the mRNA levels of inducible nitric oxide synthase (iNOS), IL-6, MMP2, and MMP9 in the BMDMs of each group were measured.

SMCs were isolated as previously described[18] and cultured using the supernatants from the cultures described previously. After treatment with Ang II for 12 hours, the B-cell lymphoma-2 (Bcl2)-associated X protein (Bax) and Bcl2 mRNA levels were investigated in the SMCs of each group.

Quantitative polymerase chain reaction

Total mRNA was collected after cells were lysed using TRIzol reagent, and cDNA was synthesized using 2 μg of total mRNA and a reverse transcription kit. Polymerase chain reaction amplification was performed using LightCycler 480 and SYBR Green Master Mix (all from Roche, Roche Diagnostics GmbH, Mannheim, Germany). The iNOS, IL-6, MMP2, MMP9, Bax, and Bcl2 mRNA levels were investigated, and the results were normalized against the expression levels of GAPDH. The target mRNA and quantitative polymerase chain reaction primer sequences are shown in Table 1.

Statistical analysis

Data were analyzed using SPSS 19.0 software (IBM Corp, Armonk, NY). Median and interquartile range were used to express quantitative data and compared using the Mann–Whitney *U* test. Categorical variables were expressed as n (%) and compared using the χ^2 test. Spearman correlation analysis was used to detect associations of IL-6, MMP2, and MMP9 with TSP-1. To identify independent predictors of the presence of acute AD, simple linear regression analyses and subsequent multivariate linear regression analyses were performed. A P value < 0.05 was considered indicative of statistical significance.

Results

Clinical characteristics of patients who provided aortic tissues

Among the patients who provided the aortic tissue, higher heart rate (HR), D-dimer levels, white blood cell (WBC) count, and C-reactive protein (CRP) levels were observed in the AD group compared with those of the control group. There were no differences in age, sex, uncontrolled or failure to control blood pressure, fasting blood glucose (Glu), creatinine,

Table 2. Characteristics of patients who provided aortic tissues

Characteristic	NAD	AD	*P*
M/F Sex, n	5/5	9/3	0.378
Age, years	44.5 (37-61)	50.50 (32.75-64.25)	0.722
SBP, mm Hg	137 (125-163)	156 (144-164)	0.093
DBP, mm Hg	90 (83-111)	95 (79-98)	0.771
HR, bpm	58 (57-74)	74 (69-84)	0.014
Glu, mmol/L	5.21 (57.00-74.25)	5.13 (4.83-5.71)	0.821
CREA (μmol/l)	68 (52.75-74.00)	69.50 (58.00-74.00)	0.479
D-dimer, μg/L	0.91 (0.71-1.02)	6.96 (3.27-10.20)	0.000
TC, mmol/L	4.48 (3.94-5.01)	4.22 (3.77-4.67)	0.283
TG, mmol/L	1.39 (0.98-1.94)	0.92 (0.80-1.19)	0.093
LDL-C, mmol/L	1.80 (1.37-2.76)	2.42 (1.54-2.95)	0.346
HDL-C, mmol/L	1.88 (1.40-2.01)	1.70 (1.11-2.17)	0.821
WBC count, $\times 10^9$/L	5.84 (4.44-6.65)	14.01 (9.56-15.16)	0.000
CRP, mg/L	0.79 (0.23-2.22)	6.06 (1.88-9.53)	0.004
Smoking, n (%)	3 (30)	7 (58.33)	0.231
HBP, n (%)	4 (40)	10 (83.33)	0.074

Data are presented as mean (interquartile range) or n (%) except where otherwise specified.

AD, aortic dissection; bpm, beats per minute; CREA, creatinine; CRP, C-reactive protein; DBP, diastolic blood pressure; F, female; Glu, fasting blood glucose; HBP, uncontrolled or failure to control blood pressure; HDL-C, high-density lipoprotein cholesterol; HR, heart rate; LDL-C, low-density lipoprotein cholesterol; M, male; NAD, non-aortic dissection; SBP, systolic blood pressure; TC, total cholesterol; TG, total triglycerides; WBC, white blood cell.

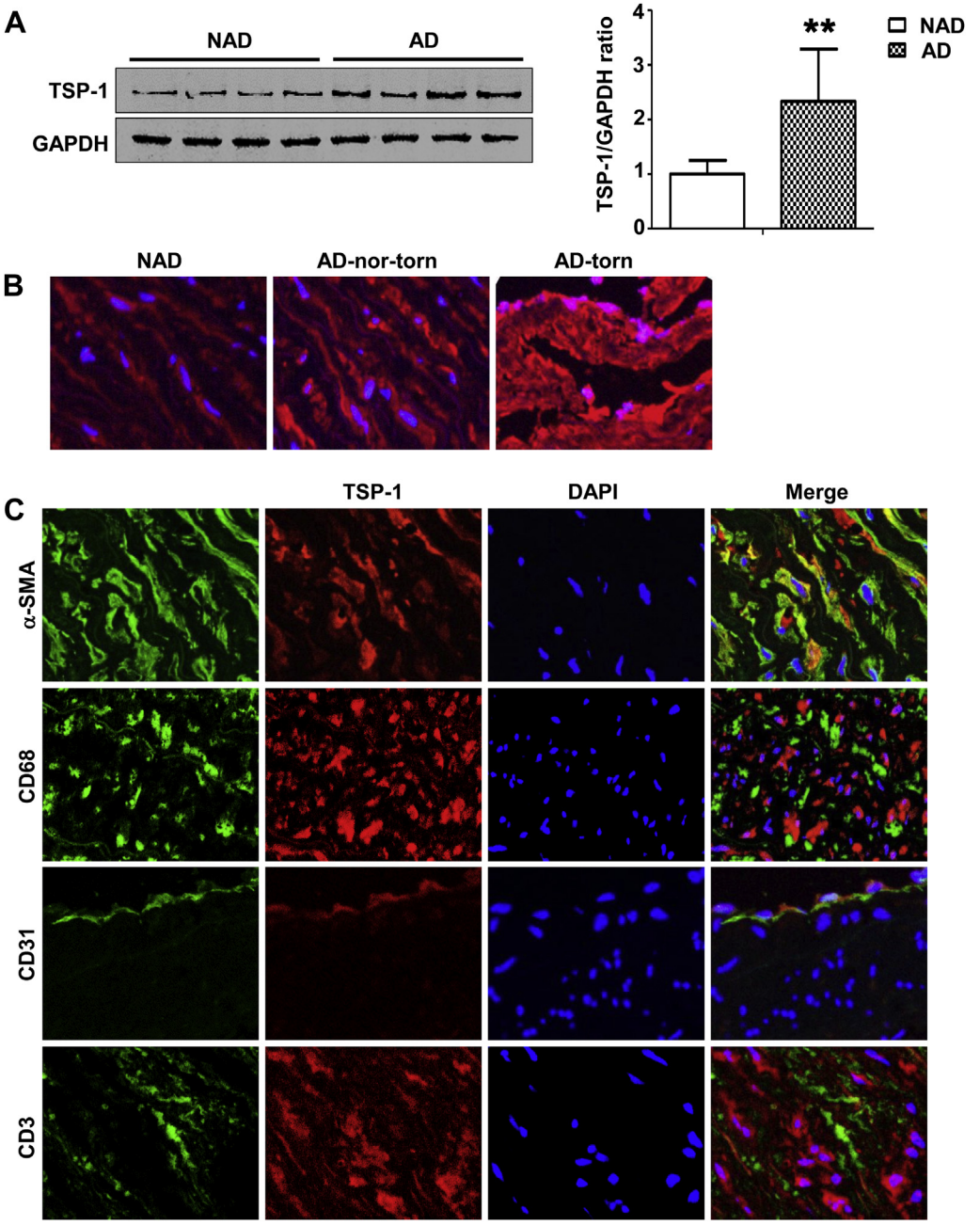

Figure 1. Thrombospondin 1 (TSP-1) expression in aortic dissection (AD) patients. (**A**) The TSP-1 levels in normal aortas and AD aortas were measured using Western blot analysis; n = 6-8 for each group, ** $P < 0.05$ vs non-AD (NAD) group. (**B**) The TSP-1 levels in these 2 groups were detected using immunofluorescence staining (magnification 200×); n = 6-8 for each group. (**C**) Double immunofluorescence staining with combinations of anti-alpha smooth muscle actin (α-SMA antibody and anti-TSP-1) antibody, anti-CD68 antibody and anti-TSP-1 antibody, anti-CD31 antibody and anti-TSP-1 antibody, and anti-CD3 antibody and anti-TSP-1 antibody in aortas from AD patients (magnification 200×); n = 6-8 for each group. DAPI, 2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

lipid levels, or smoking habits between the 2 groups. The clinical characteristics of all patients are listed in Table 2.

The expression of TSP-1 in human aortas

Western blot analysis and immunofluorescence staining were used to measure TSP-1 expression in each sample. The Western blot results showed that TSP-1 expression was significantly increased in the AD group compared with that in the control group (Fig. 1A). Immunofluorescence staining showed that the AD group exhibited higher TSP-1 levels than the control group, especially in the torn sections (Fig. 1B). In addition, double immunofluorescence staining showed that SMCs and endothelial cells, but not macrophages or T lymphocytes, were the sources of TSP-1, with SMCs being the primary source (Fig. 1C).

Table 3. Clinical characteristics of patients who provided peripheral blood

Characteristic	NAD	AD	Stanford A	Stanford B
M/F Sex, n	23/8	68/18	35/9	33/9
Age, years	64 (50-76)	56 (50-64)	55 (47-60)*	62 (53-69)
Glu, mmol/L	5.95 (5.32-6.99)	7.10 (6.37-8.30)*	7.30 (6.40-8.28)*	6.85 (6.3-8.35)*
SBP, mm Hg	156 (146-170)	145 (126-166)	141 (120. 160)*	152 (132-169)
DBP, mm Hg	78 (71-91)	78 (70-90)	75 (64-90)	80 (70-90)
HR, beats per minute	75 (68-89)	79 (68-90)	78 (60-87)	80 (72-91)
WBC count, $\times 10^9$/L	7.81 (6.26-9.51)	10.53 (7.94-13.01)*	11.54 (9.79-13.77)*	8.71 (6.63-12.53)
CRP, mg/L	3.24 (1.23-9.11)	10.74 (3.05-54.56)*	11.59 (2.95-50.20)*	9.17 (3.00-63.50)*
CREA, μmol/L	69 (59-75)	84 (69-106)*	86 (69.50-105.00)	82 (69-108)
D-dimer, μg/mL	1.56 (1.01-2.01)	4.38 (2.33-7.63)*	3.90 (1.88-6.57)*	4.68 (2.39-7.93)*
TC, mmol/L	4.01 (3.50-4.26)	4.08 (3.47-4.66)	4.16 (3.63-4.69)	4.00 (3.38-4.57)
TG, mmol/L	1.35 (1.16-1.64)	1.16 (0.88-1.77)	1.26 (0.93-1.94)	1.08 (0.83-1.58)
HDL-C, mmol/L	0.98 (0.85-1.14)	1.09 (0.87-1.36)	1.03 (0.82-1.28)	1.17 (0.91-1.470)
LDL-C, mmol/L	2.09 (1.89-2.30)	2.20 (1.66-2.69)	2.29 (1.73-2.76)	2.01 (1.61-2.69)
HBP, n (%)	25 (80.64)	76 (88.37)	39 (88.64)	37 (88.10)
Smoking, n (%)	6 (19.35)	36 (41.86)*	18 (40.91)	18 (42.86)*

Data are presented as mean (interquartile range) or n (%) except where otherwise specified.

AD, aortic dissection; bpm, beats per minute; CREA, creatinine; CRP, C-reactive protein; DBP, diastolic blood pressure; F, female; Glu, fasting blood glucose; HBP, uncontrolled or failure to control blood pressure; HDL-C, high-density lipoprotein cholesterol; HR, heart rate; LDL-C, low-density lipoprotein cholesterol; M, male; NAD, non-aortic dissection; SBP, systolic blood pressure; TC, total cholesterol; TG, total triglycerides; WBC, white blood cell.

* $P < 0.05$ vs NAD group.

Clinical characteristics of patients who provided blood samples

Glu, WBC, CRP, creatinine, D-dimer levels, and smoking rates were significantly higher in the AD group than in the NAD group. No differences in other characteristics were observed between the 2 groups, including sex, age, HR, lipid levels, or uncontrolled or failure to control blood pressure. The clinical characteristics are listed in Table 3.

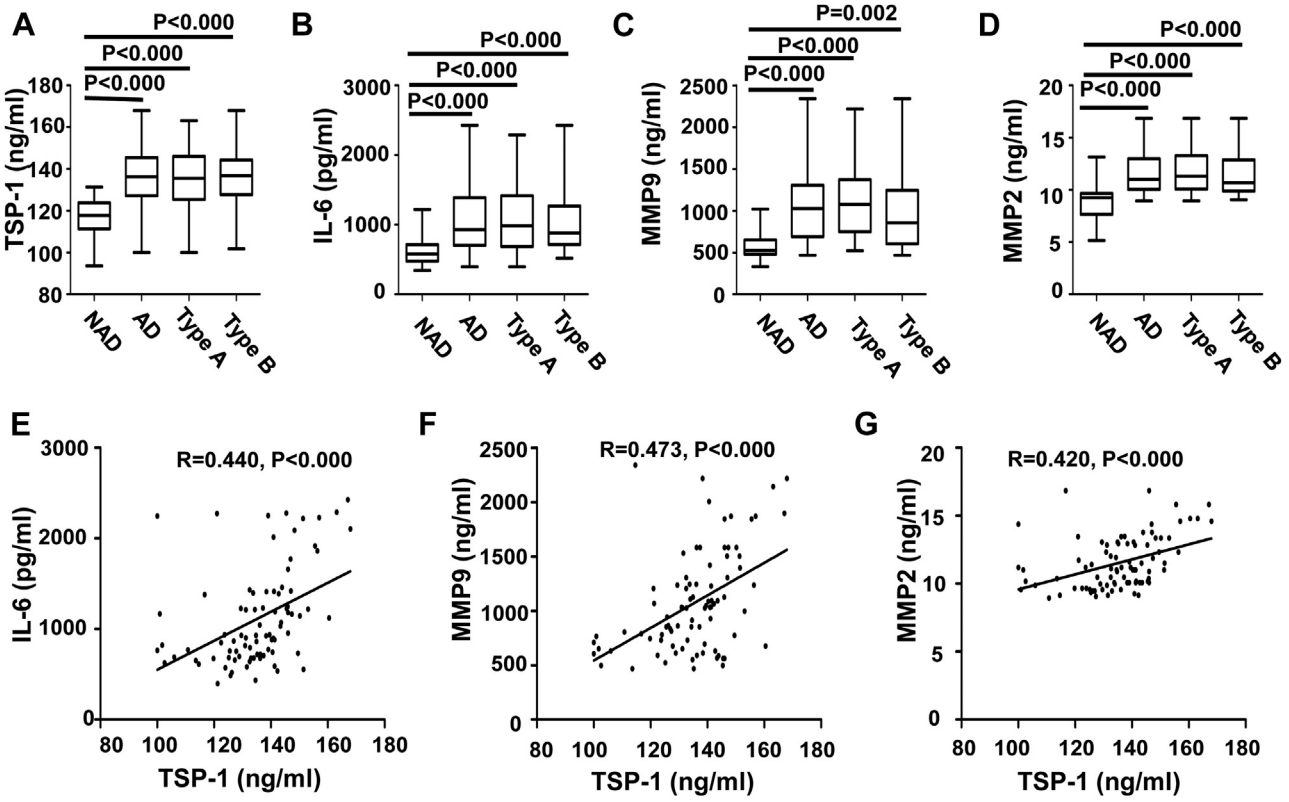

Figure 2. The plasma thrombospondin 1 (TSP-1), interleukin (IL)-6, matrix metalloproteinase (MMP)2, and MMP9 levels in the aortic dissection (AD), non-AD (NAD), Stanford A, and Stanford B groups. (**A-D**) The levels of these cytokines in each group were measured using enzyme-linked immunosorbent assay. Correlations between TSP-1 and IL-6 (**E**), MMP9 (**F**), and MMP2 (**G**) in AD patients.

Table 4. Plasma TSP-1, IL-6, MMP2, and MMP9 levels in each group

	NAD	AD	Type A	Type B
TSP-1, ng/mL	118 (111-124)	136 (127-145)*	136 (125-146)*	137 (128-144)*
IL-6, pg/mL	581 (477-714)	928 (704-1390)*	984 (685-1417)*	882 (717-1268)*
MMP9, ng/mL	527 (481-650)	1027 (690-1305)*	1077 (750-1372)*	858 (606-858)*
MMP2, ng/mL	9.26 (7.66-9.66)	11.00 (10.05,12.98)*	11.31 (10.07,13.28)*	10.69 (9.89-12.87)*

Data are presented as mean (range) or n (%) except where otherwise specified.
AD, aortic dissection; IL, interleukin; MMP, matrix metalloproteinase; NAD, non-aortic dissection; TSP-1, thrombospondin 1.
* $P < 0.05$ vs NAD group.

TSP-1, IL-6, MMP2, and MMP9 levels in AD patients

The plasma TSP-1, IL-6, MMP2, and MMP9 levels were measured using enzyme-linked immunosorbent assay. The results showed that compared with the NAD group, the AD group exhibited higher TSP-1, IL-6, MMP2, and MMP9 levels (Fig. 2A-D), whereas no differences in these cytokine levels were found between the Stanford A and Stanford B groups (Fig. 2A-D). The concentrations of these cytokines in each group are listed in Table 4. In addition, the correlation analysis showed that TSP-1 levels were positively correlated with IL-6, MMP2, and MMP9 levels in the AD group (Fig. 2E-G).

Simple linear regression analysis and multivariate linear regression analysis

Simple linear regression analysis and multivariate linear regression were performed to investigate whether TSP-1 was a risk factor for the occurrence of AD. The simple linear regression showed that TSP-1, IL-6, MMP2, MMP9, D-dimers, CRP, Glu, WBC count, and smoking status showed a trend toward the occurrence of AD ($P < 0.05$). There was no obvious trend toward occurrence of AD relative to age, creatinine, lipids, HR, or blood pressure. Thus, TSP-1, IL-6, MMP2, MMP9, D-dimers, CRP, WBC count, Glu, and smoking status were used to performed multivariate linear regression analysis. The results showed that TSP-1 ($\beta = 0.222$; 95% confidence interval [CI], 0.0200.4-24; $P = 0.032$), MMP9 ($\beta = 0.194$; 95% CI, 0.002-0.387; $P = 0.048$), MMP2 ($\beta = 0.204$; 95% CI, 0.008-0.399; $P = 0.041$), D-dimers ($\beta = 0.201$; 95% CI, 0.015-0.387; $P = 0.034$), and smoking status ($\beta = 0.186$; 95% CI, 0.036-0.036; $P = 0.016$) were independent risk factors for the occurrence of AD. The results are listed in Table 5.

Effect of rmTSP-1 on M1 macrophage differentiation and SMC apoptosis

The quantitative polymerase chain reaction results showed that TSP-1 had no effect on Ang II-treated macrophage expression of iNOS without CD4 TCs, whereas rmTSP-1 treatment further promoted Ang II and CD4 TCs-induced iNOS expression (Fig. 3A). A similar trend was observed for IL-6, MMP2, and MMP9 mRNA levels among the 4 groups (Fig. 3A). In addition, the supernatant from the BMDMs and CD4 TCs and Ang II and rmTSP-1 group culture further increased the Bax mRNA levels but reduced the Bcl2 mRNA levels in SMCs (Fig. 3B).

Discussion

In the present study, we found that TSP-1 levels were significantly increased in aortas and plasma from patients with AD. The dissected section of the aorta exhibited higher TSP-1 levels compared with the nondissected section. In addition,

Table 5. Association between cytokines, clinical characteristics, and the presence of AD were assessed using simple linear analysis and subsequent multivariate linear regression analysis

	Univariate			Multivariate		
Variables	β	95% CI	*P*	β	95% CI	*P*
TSP-1	0.515	0.356-0.673	0.000	0.222	0.020-0.424	0.032
MMP2	0.527	0.370-0.684	0.000	0.204	0.008-0.399	0.041
MMP9	0.477	0.315-0.640	0.000	0.194	0.002-0.387	0.048
Smoking	0.207	0.026-0.388	0.025	0.186	0.036-0.336	0.016
IL-6	0.419	0.251-0.586	0.000	0.033	0.735 to −0.161	0.735
D-dimer	0.478	0.316-0.640	0.000	0.201	0.015-0.387	0.034
CRP	0.259	0.005-0.081	0.005	0.08	−0.104 to 0.215	0.491
WBC	0.309	0.133-0.485	0.001	−0.061	−0.237 to 0.114	0.490
Glu	0.245	0.066-0.424	0.008	−0.055	−0.224 to 0.115	0.524
HBP	0.099	−0.085 to 0.283	0.287			
TC	0.061	−0.124 to 0.245	0.516			
TG	−0.047	−0.231 to 0.138	0.615			
LDL-C	0.071	−0.113 to 0.256	0.445			
HDL-C	0.038	−0.147 to 0.222	0.687			
CREA	0.141	−0.042 to 0.324	0.130			
HR	0.036	−0.148 to 0.221	0.698			
Age	−0.134	−0.317 to 0.049	0.149			

CI, confidence interval; CREA, creatinine; CRP, C-reactive protein; Glu, fasting blood glucose; HBP, uncontrolled or failure to control blood pressure; HDL-C, high-density lipoprotein cholesterol; HR, heart rate; IL, interleukin; LDL-C, low-density lipoprotein cholesterol; MMP, matrix metalloproteinase; TC, total cholesterol; TG, total triglycerides; TSP-1, thrombospondin 1; WBC, white blood cell.

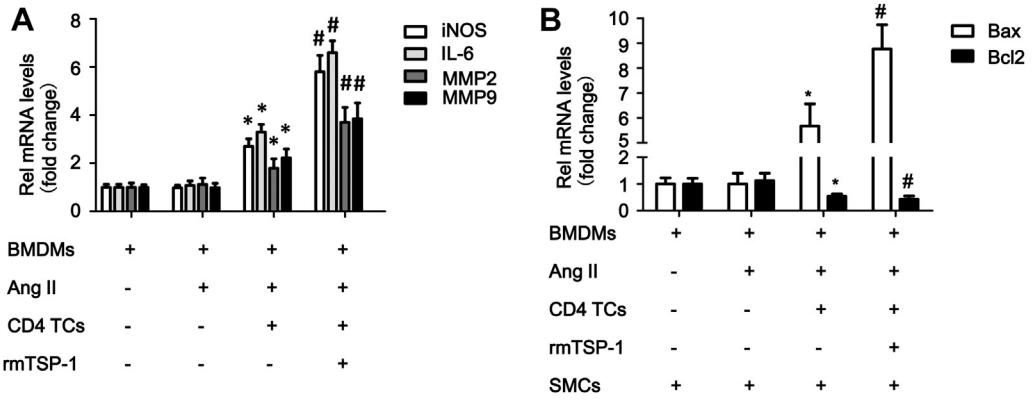

Figure 3. Effect of recombinant mouse thrombospondin 1 (rmTSP-1) on macrophage differentiation and smooth muscle cell (SMC) apoptosis. (**A**) The effect of rmTSP-1 on iNOS, interleukin (IL)-6, matrix metalloproteinase (MMP)2, and MMP9 mRNA levels in Ang II-treated macrophages was detected. (**B**) The effect of rmTSP-1 on Bax and Bcl2 mRNA levels in Ang II-treated SMCs was measured. n = 4-5 in each group. * $P < 0.05$ vs the Bone marrow-derived macrophages (BMDMs) group and BMDMs and Ang II group; # $P < 0.05$ vs the BMDMs with CD4 T-lymphocytes (TCs) and Ang II group. Ang, angiotensin; Bax, Bcl2-associated X protein; Bcl2, B-cell lymphoma-2; iNOS, inducible nitric oxide synthase.

SMCs were the main source of TSP-1 in the aortas of AD patients, and endothelial cells also secreted small amounts of TSP-1. Circulating TSP-1 levels were positively correlated with the IL-6, MMP2, and MMP9 levels in AD patients, and TSP-1 was independently associated with the occurrence of AD. rmTSP-1 promoted Ang II-induced iNOS mRNA expression in macrophages and further increased Bax mRNA levels and reduced Bcl2 mRNA levels in SMCs, supporting proapoptotic activity.

In a previous study, a large number of inflammatory mediators and cells were observed in the blood vessel walls of AD. In addition, the inflammatory mediators and cells were not fixed and changed according to the progression of AD,[19,20] suggesting that the imbalance between proinflammation and anti-inflammation could contribute to AD. TSP-1 is an important ECM protein that could participate in many diseases via regulating inflammatory responses. In an earlier study, Puxeddu et al. reported that TSP-1 levels were increased in the sera of patients with chronic spontaneous urticaria.[21] In another study, higher TSP-1 levels were observed in acute ischemic stroke patients and had a predictive value for the outcomes in these patients.[22] In addition, TSP-1 levels were also reported to be increased in cardiovascular diseases, such as ST-segment elevation myocardial infarction with plaque erosion.[15] In our study, we observed higher TSP-1 levels in aortas and plasma from patients with AD, and aortic TSP-1 expression was further increased in the torn section of aortas from AD patients. The results suggest that TSP-1 might be involved in the occurrence of AD.

Among the numerous inflammatory cells, macrophages are most closely related to AD[19,23] because they can release high levels of inflammatory substances, resulting in excessive loss of SMCs, reduction in extracellular mechanisms, and the occurrence of AD.[19,24] Notably, macrophages can secrete multiple cytokines, such as IL-6, IL-17, and MMPs, which have been shown to be closely related to the occurrence of AD. Among these cytokines, IL-6 was increased in human and mouse AD.[23,25,26] IL-6 upregulated the activation of the signal transducer and activator of transcription 3 (STAT3) pathway, promoted helper T lymphocyte 17 (Th17) differentiation, and macrophage infiltration in the vessel wall, and aggravated Ang II-induced mouse AD, whereas deletion of IL-6 significantly protected against SMC dysfunction and alleviated the occurrence of AD.[27,28] Ishii and Asuwa reported that the expression of MMP2 and MMP9 in vascular SMCs was significantly higher in the degenerated media compared with other regions in AD, suggesting that MMP2 and MMP9 overexpression in SMCs is involved in AD formation by promoting ECM degradation.[29] In a later article, Kurihara et al. reported that MMP9 was most closely related to AD, and that MMP9 significantly promoted the loss of SMCs, whereas deletion of MMP9 could significantly reduce the incidence of AD.[30] To explore the association of TSP-1 with inflammation, we quantified the plasma IL-6, MMP2, and MMP9 levels and found that these 3 factors were increased in AD patients and positively correlated with TSP-1 levels. These results suggest that TSP-1 participates in the occurrence of AD via regulating macrophage-mediated inflammation.

Classically activated macrophages (M1 macrophages) and alternatively activated macrophages (M2 macrophages) play proinflammatory and anti-inflammatory roles, respectively. In the early stage of the inflammatory response, M1 macrophage levels are increased whereas M2 macrophage levels are reduced; at later stages, M1 macrophage and M2 macrophage levels are increased, with the anti-inflammatory effect of M2 macrophages being unable to counteract the proinflammatory role of M1 macrophages.[23,24] These data suggest that M1 macrophages contribute significantly to the occurrence of AD. Therefore, to investigate the role of TSP-1 in macrophage differentiation, rmTSP-1 was used to stimulate Ang II-treated BMDMs. Because a previous study showed that CD4 TCs were important for the differentiation of macrophages,[16] some BMDMs were cocultured with CD4 TCs. Interestingly, the results showed that rmTSP-1 treatment significantly increased iNOS mRNA levels in Ang II-treated BMDMs, together with similar trends for IL-6, MMP2, and MMP9 mRNA levels. These results show that rmTSP-1 could promote Ang II-induced M1 macrophage differentiation in vitro and that TSP-1 might be involved in AD via promoting macrophage differentiation and aggravating SMC apoptosis.

The aorta is composed of 3 layers: the endothelium, the media composed of elastin and SMCs, and the adventitia containing fibroblasts. SMCs are involved in the regulation of the ECM and are important for maintaining the normal structure and function of blood vessels.[31-33] Previous studies have shown that excessive apoptosis of vascular SMCs can lead to hemangiomatoid dilation and even acute AD.[34] To confirm the speculation and further explore the mechanisms, the effect of TSP-1 on SMC apoptosis was tested and results showed that Bax mRNA levels were increased in SMCs whereas Bcl2 mRNA levels were reduced by rmTSP-1 treatment. These results suggest that rmTSP-1 could aggravate Ang II-induced SMC apoptosis, promote M1 macrophage differentiation, mediate SMC excessive apoptosis, and participate in the progression of AD.

In summary, we found that the involvement of TSP-1 in AD might be due to the differentiation of M1 macrophages and apoptosis of SMCs.

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Disclosures

The authors have no conflicts of interest to disclose.

References

1. Cifani N, Proietta M, Tritapepe L, et al. Stanford-A acute aortic dissection, inflammation, and metalloproteinases: a review. Ann Med 2015;47:441-6.
2. Milewicz DM. Stopping a killer: improving the diagnosis, treatment, and prevention of acute ascending aortic dissections. Circulation 2011;124:1902-4.
3. Golledge J, Muller J, Daugherty A, Norman P. Abdominal aortic aneurysm: pathogenesis and implications for management. Arterioscler Thromb Vasc Biol 2006;26:2605-13.
4. McLaren KM. Immunohistochemical localisation of thrombospondin in human megakaryocytes and platelets. J Clin Pathol 1983;36:197-9.
5. Zhao Y, Xiong Z, Lechner EJ, et al. Thrombospondin-1 triggers macrophage IL-10 production and promotes resolution of experimental lung injury. Mucosal Immunol 2014;7:440-8.
6. Velasco P, Huegel R, Brasch J, et al. The angiogenesis inhibitor thrombospondin-1 inhibits acute cutaneous hypersensitivity reactions. J Invest Dermatol 2009;129:2022-30.
7. Rogers NM, Ghimire K, Calzada MJ, Isenberg JS. Matricellular protein thrombospondin-1 in pulmonary hypertension: multiple pathways to disease. Cardiovasc Res 2017;113:858-68.
8. Gomez D, Coyet A, Ollivier V, et al. Epigenetic control of vascular smooth muscle cells in Marfan and non-Marfan thoracic aortic aneurysms. Cardiovasc Res 2011;89:446-56.
9. Gomez D, Kessler K, Michel JB, Vranckx R. Modifications of chromatin dynamics control Smad2 pathway activation in aneurysmal smooth muscle cells. Circ Res 2013;113:881-90.
10. Meijles DN, Sahoo S, Al Ghouleh I, et al. The matricellular protein TSP1 promotes human and mouse endothelial cell senescence through CD47 and Nox1. Sci Signal 2017;10:eaaj1784.
11. Kim CW, Pokutta-Paskaleva A, Kumar S, et al. Disturbed flow promotes arterial stiffening through thrombospondin-1. Circulation 2017;136:1217-32.
12. Zhao XM, Hu Y, Miller GG, Mitchell RN, Libby P. Association of thrombospondin-1 and cardiac allograft vasculopathy in human cardiac allografts. Circulation 2001;103:525-31.
13. Wu X, Luo X, Zhu Q, et al. The roles of thrombospondins in hemorrhagic stroke. Biomed Res Int 2017;2017:8403184.
14. Krishna MS, Seto SW, Jose R, et al. High serum thrombospondin-1 concentration is associated with slower abdominal aortic aneurysm growth and deficiency of thrombospondin-1 promotes angiotensin II induced aortic aneurysm in mice. Clin Sci (Lond) 2017;131:1261-81.
15. Chandran S, Watkins J, Abdul-Aziz A, et al. Inflammatory differences in plaque erosion and rupture in patients with ST-segment elevation myocardial infarction. J Am Heart Assoc 2017;6:e005868.
16. Li Y, Zhang C, Wu Y, et al. Interleukin-12p35 deletion promotes CD4 T-cell-dependent macrophage differentiation and enhances angiotensin II-Induced cardiac fibrosis. Arterioscler Thromb Vasc Biol 2012;32:1662-74.
17. Lake FR, Noble PW, Henson PM, Riches DW. Functional switching of macrophage responses to tumor necrosis factor-alpha (TNF alpha) by interferons. Implications for the pleiotropic activities of TNF alpha. J Clin Invest 1994;93:1661-9.
18. Linde CI, Antos LK, Golovina VA, Blaustein MP. Nanomolar ouabain increases NCX1 expression and enhances Ca2+ signaling in human arterial myocytes: a mechanism that links salt to increased vascular resistance? Am J Physiol Heart Circ Physiol 2012;303:H784-94.
19. Son BK, Sawaki D, Tomida S, et al. Granulocyte macrophage colony-stimulating factor is required for aortic dissection/intramural haematoma. Nat Commun 2015;6:6994.
20. Tieu BC, Lee C, Sun H, et al. An adventitial IL-6/MCP1 amplification loop accelerates macrophage-mediated vascular inflammation leading to aortic dissection in mice. J Clin Invest 2009;119:3637-51.
21. Puxeddu I, Rabl SC, Panza F, et al. Endostatin and thrombospondin-1 levels are increased in the sera of patients with chronic spontaneous urticaria. Arch Dermatol Res 2014;306:197-200.
22. Gao JB, Tang WD, Wang HX, Xu Y. Predictive value of thrombospondin-1 for outcomes in patients with acute ischemic stroke. Clin Chim Acta 2015;450:176-80.
23. Anzai A, Shimoda M, Endo J, et al. Adventitial CXCL1/G-CSF expression in response to acute aortic dissection triggers local neutrophil recruitment and activation leading to aortic rupture. Circ Res 2015;116:612-23.
24. Zhang P, Hou S, Chen J, et al. Smad4 Deficiency in smooth muscle cells initiates the formation of aortic aneurysm. Circ Res 2016;118:388-99.
25. Brasier AR. The nuclear factor-kappaB-interleukin-6 signalling pathway mediating vascular inflammation. Cardiovasc Res 2010;86:211-8.
26. Ye J, Wang M, Jiang H, et al. Increased levels of interleukin-22 in thoracic aorta and plasma from patients with acute thoracic aortic dissection. Clin Chim Acta 2018;486:395-401.
27. Ju X, Ijaz T, Sun H, et al. Interleukin-6-signal transducer and activator of transcription-3 signaling mediates aortic dissections induced by angiotensin II via the T-helper lymphocyte 17-interleukin 17 axis in C57BL/6 mice. Arterioscler Thromb Vasc Biol 2013;33:1612-21.
28. An Z, Qiao F, Lu Q, et al. Interleukin-6 downregulated vascular smooth muscle cell contractile proteins via ATG4B-mediated

autophagy in thoracic aortic dissection. Heart Vessels 2017;32:1523-35.

29. Ishii T, Asuwa N. Collagen and elastin degradation by matrix metalloproteinases and tissue inhibitors of matrix metalloproteinase in aortic dissection. Hum Pathol 2000;31:640-6.

30. Kurihara T, Shimizu-Hirota R, Shimoda M, et al. Neutrophil-derived matrix metalloproteinase 9 triggers acute aortic dissection. Circulation 2012;126:3070-80.

31. Xu J, Shi GP. Vascular wall extracellular matrix proteins and vascular diseases. Biochim Biophys Acta 2014;1842:2106-19.

32. El-Hamamsy I, Yacoub MH. Cellular and molecular mechanisms of thoracic aortic aneurysms. Nat Rev Cardiol 2009;6:771-86.

33. Michel JB, Jondeau G, Milewicz DM. From genetics to response to injury: vascular smooth muscle cells in aneurysms and dissections of the ascending aorta. Cardiovasc Res 2018;114:578-89.

34. He R, Guo DC, Estrera AL, et al. Characterization of the inflammatory and apoptotic cells in the aortas of patients with ascending thoracic aortic aneurysms and dissections. J Thorac Cardiovasc Surg 2006;131:671-8.