Thrombospondin-1 Activates Medial Smooth Muscle Cells and Triggers Neointima Formation Upon Mouse Carotid Artery Ligation

Rute Moura, Marc Tjwa, Petra Vandervoort, Katrien Cludts, Marc F. Hoylaerts

Objective—Thrombospondin-1 (TSP1) is described as a positive regulator of vascular smooth muscle growth in cell culture. However, insight into the in vivo effects of TSP1 on smooth muscle cell (SMC) function is lacking.

Methods and Results—We analyzed wild-type (WT) and TSP1-deficient (Tsp1<sup>−/−</sup>) mice in a carotid artery ligation model, in which neointimal lesions form without overt mechanical damage to the endothelium. On ligation, the expression of TSP1 increased strongly in the matrix of neointima and adventitia. In the early phase after ligation (day 3 to 7), activation, proliferation, and migration of medial SMCs were delayed and impaired in Tsp1<sup>−/−</sup> mice, in parallel with defective upregulation of metalloproteinase (MMP)-2 activity. As a result, Tsp1<sup>−/−</sup> arteries developed smaller neointimal lesions, a thicker media but comparably attenuated patency as in WT arteries, 28 days after ligation. Furthermore, medial and neointimal SMCs in Tsp1<sup>−/−</sup> mice produced more collagen, more osteopontin, and displayed weaker smooth muscle actin staining than WT SMCs, indicative of a modified SMC phenotype in Tsp1<sup>−/−</sup> mice.

Conclusions—Arterial SMC activation in the absence of TSP1 is delayed and dysregulated, reducing neointima formation, on mild vascular injury. (Arterioscler Thromb Vasc Biol. 2007;27:2163-2169.)

Key Words: carotid artery ■ matricellular proteins ■ neointima ■ smooth muscle cells

Thrombospondin-1 (TSP1) is a matricellular protein, with a complex multidomain structure which can interact with a great variety of receptors, ligands, and matrix components (reviewed in<sup>1-2</sup>). It is highly expressed during development, but present at a low level in adult tissues and upregulated in response to injury.<sup>3-5</sup> TSP1 is present in several cell types, including vascular cells, from which it is secreted on cell activation to participate in fine modulation of cell-cell and cell-matrix interactions and regulation of cell function.<sup>6-8</sup> TSP1 gene-deficient (Tsp1<sup>−/−</sup>) mice are viable, and apparently normal, but challenge of these mice in various models has shown that TSP1 inhibits angiogenesis,<sup>9,10</sup> promotes wound healing in an excisional wound healing model,<sup>11</sup> and contributes to pulmonary homeostasis.<sup>12</sup> We have recently shown an in vivo role for TSP1 in platelet and thrombus adhesion to the injured vessel wall.<sup>13</sup>

Percutaneous coronary interventions often result in the occurrence of intimal hyperplasia and restenosis.<sup>14</sup> They are triggered by endothelial injury, and involve proliferation and migration of smooth muscle cells (SMCs) from the media into the intima. A role for TSP1 in vascular injury has been suggested by the strong TSP1 upregulation in rat carotid arteries after balloon-angioplasty<sup>15</sup> and at sites of hyperplasia, in hypercholesterolemic lesions, and in the adventitia of diabetic rats.<sup>5,16,17</sup>

Although TSP1 is not essential for SMC growth in vitro, the treatment of SMCs with growth factors results in strong TSP1 expression, which further amplifies SMC proliferation.<sup>18-21</sup> The inhibition of TSP1 by specific antibodies reduced SMC proliferation.<sup>21</sup> TSP1 also acts as a chemottractant for SMCs via interactions between its carboxyl terminus and CD47, and hence integrins.<sup>22-26</sup> In addition, Tsp1<sup>−/−</sup> SMCs show selective defects in proliferation and chemotaxis responses to PDGF.<sup>27</sup> The TSP1-induced SMC migration on gelatin appears to rely on metalloproteinase (MMP)-2 activation.<sup>28,29</sup> Indeed, endogenous TSP1 increases MMP2 levels and limits collagen production in the vascular outgrowth of muscle explants.<sup>29</sup>

In vivo, TSP1 neutralization by antibodies resulted in less neointima development, after carotid artery denudation,<sup>30</sup> but it remains unclear to which extent this was attributable to accelerated reendothelialization or decreased vascular SMC function. Nevertheless, the study in Tsp1<sup>−/−</sup> mice of healing myocardial infarcts has revealed a role for TSP1 in protecting the noninfarcted myocardium from fibrotic remodeling.<sup>31</sup> In experimental models of ischemic injury, Tsp1<sup>−/−</sup> mice show an increased nitric oxide (NO)-mediated tissue perfusion, implicating TSP1 as a physiological antagonist of NO-mediated vasodilatation and tissue perfusion. Elegant work...
has shown that CD47 is the TSP1 receptor, limiting the NO-mediated vascular smooth muscle relaxation.32

In this study, by comparing WT and Tspo−/− mice, we aimed at studying the specific role of TSP1 in SMC activation and in phenotype switching from contractile to synthetic SMCs, after mild vascular injury. Therefore, we have used an established model of carotid artery ligation,33 in which, after an early phase of inflammatory cell recruitment, medial SMCs rapidly proliferate and migrate toward the lumen, leading to extensive neointima formation after 4 weeks, without involving direct damage to the endothelium.

Methods

Model of Mouse Carotid Artery Ligation

All animal experiments were reviewed and approved by the Institutional Review Board of the University of Leuven and were performed in compliance with the guidelines of the International Society on Thrombosis and Hemostasis.34 Tspo−/− mice12 were kindly provided by Dr J. Lawler (Harvard, Boston, Mass). Male Tspo−/− and WT littermate mice, aged 12 to 24 weeks and bred for more than 8 generations into the C57Bl/6 background were used in this study. The carotid artery ligation model has been previously described.33 Briefly, the left common carotid artery was dissected free of connective tissue and permanently ligated proximally to the bifurcation. At specified time points after surgery, animals were euthanized, perfused, and paraffin serial 7-μm-thick sections were prepared, covering the area 5 mm proximal to the ligation site. The area analyzed comprised a minimum of 4 cross-sections in the zone of maximal response, between 1 mm and 3 mm, distally from the ligation site. For more details on the animal model, histology, and morphometry, please see the supplemental materials (available online at http://atvb.ahajournals.org).

SMC Assessment and Proliferation

The number of nuclear profiles per power field in the media was counted on H&E stainings to assess loss and recovery of medial SMCs after ligation. Results were expressed as cell densities. Because smooth muscle actin (SMA) is a marker for contractile SMCs and immunoreactivity to SMA is reduced in replicating, synthetic SMCs, SMA staining was used to evaluate the SMC phenotype. Cell proliferation was measured by immunostaining for proliferating cell nuclear antigen (PCNA). Proliferation rates were expressed as a percentage of PCNA-stained nuclei/total nuclei.

Immunoblot Analysis

Vascular extracts of ligated carotid artery (0.5 to 10 μg of total protein) were subjected to SDS-PAGE. Blots were revealed via indirect staining, using antibodies against SMC actin (1/1000 Dako SMO651), OPN (1/1000 Santa Cruz Biotechnology, sc-10593), and TSP1 (50 μg/mL home-made Rabbit-anti-human TSP1). Staining was performed with the Pierce Western blotting enhanced chemiluminescence (ECL) detection reagent (Perbio).

Gelatin Zymography

Gelatin zymography was performed as described.35 Protein extracts (5 μg) of carotid arteries mixed with SDS sample buffer were loaded onto 10% gels containing 0.1% gelatin (Invitrogen, NOVEX EC6175BOX). Digestion bands were quantified using the Zeiss KS300 software. Cell extracts of HT1080 cells served as an internal reference for the identification of MMP2 and MMP9 activity bands.

Statistical Analysis

Data are represented as the mean±SD. Significance of differences was analyzed using a 2-tailed unpaired t test with Welch correction, allowing for populations with different SD. A value of P<0.05 was considered statistically significant. Time course experiments were analyzed by 1-way analysis of variance (ANOVA).

Results

Vascular Tspo Expression After Carotid Artery Ligation in Mice

In native carotid arteries of WT mice, TSP1 was faintly expressed in the adventitia, and in some areas of the endothelial lining and media (Fig 1A). At day 3 after ligation, TSP1 was upregulated in the adventitia (Figure 1B and 1C) and at day 28, the developed neointima stained strongly for TSP1 in the ECM and cells of adventitia and neointima (NI), at days 0, 3, and 28 after ligation in WT mice, as indicated; TSP1 presence in the endothelial lining, media (M), and lumen (L) is shown in detail (C); TSP1 in the media is highlighted by a black arrow (C) and in neointimal inflammatory cells by white arrows (E). Bar=50 μm.

Figure 1. TSP1 staining in ligated carotid arteries. Immunohistochemical detection of TSP1 in the ECM and cells of adventitia and neointima (NI), at days 0, 3, and 28 after ligation in WT mice, as indicated; TSP1 presence in the endothelial lining, media (M), and lumen (L) is shown in detail (C); TSP1 in the media is highlighted by a black arrow (C) and in neointimal inflammatory cells by white arrows (E). Bar=50 μm.
Carotid Artery Morphology and Neointima Development After Ligation

No morphological differences were observed in resting carotid artery cross-sections of WT and Tsp1<sup>-/-</sup> mice (Figure 2, day 0). Three days after ligation, medial SMCs in WT sections had lost their elongated profile, leading to medial thickening. This was not observed in Tsp1<sup>-/-</sup> sections (Figure 2, day 3). At day 7, medial thickening in the absence of neointima was observed in both genotypes (Figure 2, day 7), whereas at day 28, neointima had developed in both genotypes, 1.7-fold (supplemental Figure Ia) more extensively in WT than Tsp1<sup>-/-</sup> mice. These data indicated impaired neointima development and suggested delayed SMC activation, in the absence of TSP1.

Geometric Remodeling and Vascular Patency After Carotid Artery Ligation

Morphometric analysis of the ligated carotid artery revealed that the external elastic lamina area (EEL) increases beyond day 7 in WT but not in Tsp1<sup>-/-</sup> mice (supplemental Figure Ia). In WT but not in Tsp1<sup>-/-</sup> mice, medial thickening early after ligation (Figure 2), was accompanied by an increase in medial area from day 3 to 28 (supplemental Figure Ib). This occurred despite a reduction in the medial area index (medial area/ EEL area), a parameter of medial thickening, at day 28 (supplemental Figure Ic). In contrast, medial thickness was still observed in Tsp1<sup>-/-</sup> mice on day 28 (Figure 2 day 28, supplemental Figure Ic), suggestive of long-lasting impaired SMC migration.

We wished to investigate whether the larger vessel size with a thin media in WT and smaller vessel size with a thick media in Tsp1<sup>-/-</sup> mice, resulted from different eutrophic wall remodeling. We therefore studied arterial remodeling in both genotypes by plotting the EEL at day 28 as a function of the arterial area (Figure 2). This revealed a linear relationship in WT cross-sections (R<sup>2</sup>=0.87; P<0.0001; slope=0.81; y intersection=41 790) and Tsp1<sup>-/-</sup> cross-sections (R<sup>2</sup>=0.47; P=0.0002; slope=0.83; y intersection=35 050), with comparable slopes and intersections in both genotypes (supplemental Figure Ie and If). These data confirm that the relative outward eutrophic geometric remodeling in WT and Tsp1<sup>-/-</sup> mice is identical, its extent in Tsp1<sup>-/-</sup> mice being determined by the smaller neointima on day 28, in these mice (supplemental Figure Id).

The comparable luminal cross-sectional area on day 28 in WT and Tsp1<sup>-/-</sup> mice (supplemental Figure Ia), is therefore explained by less neointima formation, less outward remodeling, and a thicker media in Tsp1<sup>-/-</sup> mice.

Responses of Medial SMCs After Ligation

Ligation of the WT murine carotid artery leads to an initial loss of medial SMCs followed by SMC proliferation and recovery.33 We therefore quantified, by counting the nuclei per high power field on H&E stained sections, the medial SMC density of WT and Tsp1<sup>-/-</sup> mice at different time points after ligation. At baseline, no genotypic differences were found in WT and Tsp1<sup>-/-</sup> mice (Figure 3A). At day 3 after ligation, WT and Tsp1<sup>-/-</sup> mice underwent comparable and significant reduction in medial SMC density. In WT mice, this drop was followed at day 7 by a 1.5-fold increase of the SMC density, and at day 28 by a secondary drop (Figure 3C). In contrast, Tsp1<sup>-/-</sup> mice seemed to fail in responding to the initial loss of SMCs up to 28 days after ligation. More detailed analysis in both genotypes of SMC proliferation by quantifying PCNA-stained nuclei in the media after ligation, showed that medial SMC proliferation was initiated around day 3, increased at day 7, and was almost back to baseline levels at day 28 (Figure 3B). Both at day 3 and day 7, the percentage of PCNA-stained cells was drastically reduced in Tsp1<sup>-/-</sup>, compared with WT mice. Thus, SMC proliferation was impaired in Tsp1<sup>-/-</sup> mice, on carotid artery ligation.

MMP2 is a marker of migrating and activated SMCs during vasculopathy.36,37 We therefore analyzed its expression by zymography using arterial extracts (supplemental Figure II). At baseline, MMP2 was hardly detectable in both genotypes (Figure 3C). In WT mice, vessel occlusion resulted in greatly increased MMP2 levels, 3 and 7 days after ligation (Figure 3C). In contrast, the increase of MMP2 levels was significantly reduced in Tsp1<sup>-/-</sup> mice (Figure 3C). Notably, these results might further corroborate the impaired migration of Tsp1<sup>-/-</sup> SMCs (see above). The smaller increase of MMP2 levels in Tsp1<sup>-/-</sup> mice was specific as the expression of MMP9, predominantly derived from infiltrated inflammatory cells,37 was not different between WT and Tsp1<sup>-/-</sup> mice.

Figure 2. Morphology of WT and Tsp1<sup>-/-</sup> carotid arteries after ligation. Representative light microscopy photos of H&E-stained carotid artery cross-sections from WT and Tsp1<sup>-/-</sup> mice, at day 0, 3, 7, and 28 after ligation, as indicated; Note medial thickening in WT mice on day 3 and 7, and in Tsp1<sup>-/-</sup> mice on day 7 and 28. Bar=50 μm.
Indeed, MMP9 levels increased after ligation in both genotypes, consistent with enhanced and similar infiltration in WT and \textit{Tsp1}/\textit{H11002}/\textit{H11002} vessels of leukocytes and macrophages, identified by CD45 and Mac3 staining, respectively (supplemental Table I).

**Loss of Contractile SMC Phenotype and Collagen Deposition in the Media**

The presence of SMA is characteristic of contractile nonactivated SMCs. We therefore used the absence of SMA as a parameter of SMC differentiation. At baseline, medial SMA staining was comparable between genotypes (supplemental Figure III). In WT mice, medial SMA staining was strongly reduced 3 days after ligation compared with baseline and remained strongly negative 7 days after ligation. In contrast, SMA staining in \textit{Tsp1}/\textit{H11002} mice was only minimally affected 3 days after ligation, and decreased significantly only at day 7 (supplemental Figure III). This finding was in agreement with the lack of medial thickening at day 3, which only occurred at day 7 (see Figure 2). These results suggest that loss of the contractile SMC phenotype in \textit{Tsp1}/\textit{H11002} mice was delayed. Consistently, medial SMA staining had returned to normal levels in WT cross-sections at day 28, but remained attenuated in \textit{Tsp1}/\textit{H11002} sections, in agreement with a different medial SMC phenotype at day 28 in \textit{Tsp1}/\textit{H11002} carotid arteries (supplemental Figure III).

Because SMCs are a predominant source of collagen in blood vessels, we analyzed the collagen content in the vessels by Sirius Red (SR) staining. At baseline, the medial SR-positive area was approximately 40% in WT and \textit{Tsp1}/\textit{H11002} mice, and only insignificantly increased 3 days after ligation (supplemental Figure IVa). At day 28 after ligation, the medial collagen content was not altered in WT mice, but was elevated 1.5-fold in the \textit{Tsp1}/\textit{H11002} media (supplemental Figure IVa). The higher collagen deposition was not evenly distributed, but rather appeared as patches throughout the \textit{Tsp1}/\textit{H11002} media (supplemental Figure IVc and IVd). Adventitial collagen analysis confirmed that TSP1 specifically controlled

![Figure 3D](image-url). Indeed, MMP9 levels increased after ligation in both genotypes, consistent with enhanced and similar infiltration in WT and \textit{Tsp1}/\textit{H11002}/\textit{H11002} vessels of leukocytes and macrophages, identified by CD45 and Mac3 staining, respectively (supplemental Table I).

**Loss of Contractile SMC Phenotype and Collagen Deposition in the Media**

The presence of SMA is characteristic of contractile nonactivated SMCs. We therefore used the absence of SMA as a parameter of SMC differentiation. At baseline, medial SMA staining was comparable between genotypes (supplemental Figure III). In WT mice, medial SMA staining was strongly reduced 3 days after ligation compared with baseline and remained strongly negative 7 days after ligation. In contrast, SMA staining in \textit{Tsp1}/\textit{H11002} mice was only minimally affected 3 days after ligation, and decreased significantly only at day 7 (supplemental Figure III). This finding was in agreement with the lack of medial thickening at day 3, which only occurred at day 7 (see Figure 2). These results suggest that loss of the contractile SMC phenotype in \textit{Tsp1}/\textit{H11002} mice was delayed. Consistently, medial SMA staining had returned to normal levels in WT cross-sections at day 28, but remained attenuated in \textit{Tsp1}/\textit{H11002} sections, in agreement with a different medial SMC phenotype at day 28 in \textit{Tsp1}/\textit{H11002} carotid arteries (supplemental Figure III).

Because SMCs are a predominant source of collagen in blood vessels, we analyzed the collagen content in the vessels by Sirius Red (SR) staining. At baseline, the medial SR-positive area was approximately 40% in WT and \textit{Tsp1}/\textit{H11002} mice, and only insignificantly increased 3 days after ligation (supplemental Figure IVa). At day 28 after ligation, the medial collagen content was not altered in WT mice, but was elevated 1.5-fold in the \textit{Tsp1}/\textit{H11002} media (supplemental Figure IVa). The higher collagen deposition was not evenly distributed, but rather appeared as patches throughout the \textit{Tsp1}/\textit{H11002} media (supplemental Figure IVc and IVd). Adventitial collagen analysis confirmed that TSP1 specifically controlled

![Figure 3](image-url). SMC function and MMP expression in the media of ligated carotid arteries. SMC density (number of nuclei counted/area in H&E staining; A); proliferation index as assessed by PCNA staining (B); gelatinase activity in protein extracts from carotid arteries harvested at the indicated time-points, analyzed by zymography and quantified by densitometry as total (pro + active form) MMP2 (C) or MMP9 (D), as a function of time, for WT (black bars) and \textit{Tsp1}/\textit{H11002} mice (white bars). The relevant bands of the zymogram are shown underneath the bars. (The complete gel is shown in supplemental Figure II.)
collagen deposition of SMCs, but not of adventitial fibroblasts, because adventitial collagen did not differ over time between genotypes (supplemental Figure IVb).

Different Neointima Phenotype in Tsp1<sup>−/−</sup> Mice

The different WT and Tsp1<sup>−/−</sup> media composition at day 28, prompted us to analyze the SMC-rich neointima in more detail. In WT mice, the mature neointima contained predominantly SMA-positive cells, with a SMA-positive area equivalent to 57±21% (n=19; Figure 4A). These data suggest that the vast majority of neointimal SMCs 28 days after ligation had reacquired the contractile nonactivated phenotype. Surprisingly, the SMA-positive area within the neointima was significantly smaller in Tsp1<sup>−/−</sup> mice (22±23%; n=19; P<0.0001 versus WT; Figure 4B), with a larger proportion of cells not staining for SMA, suggesting late dedifferentiated neointimal SMCs. The smaller SMA content was also detectable by Western blotting (Figure 4C), when equal amounts of vascular extracted proteins were compared. As for the media, the collagen content was increased in the neointimal lesion of Tsp1<sup>−/−</sup> mice (SR<sup>+</sup> area was 28±20% in WT versus 53±27% in Tsp1<sup>−/−</sup> mice; n=12; P<0.05; Figure 4D and 4E).

Because the medial SMC recovery was defective in Tsp1<sup>−/−</sup> ligated arteries (see above), we also quantified nuclei numbers per high-power field in the neointima. In WT mice, nuclear density was 5916±1963 nuclei/μm² (n=24; Figure 4F). Consistent with less proliferation and smaller lesions (see above), Tsp1<sup>−/−</sup> neointimas showed a slightly lower nuclear density (4842±1781; n=24; P=0.05 versus WT; Figure 4G), though barely significantly different. The comparably low proliferation index of 10.4±9.2% in WT versus 9.2±5.9% in Tsp1<sup>−/−</sup> neointima, confirmed that SMCs had halted to proliferate to the same degree in both genotypes.

Collagen-producing SMCs synthesize more osteopontin (OPN). Therefore, we quantified OPN immunohistochemically in the neointima of WT and Tsp1<sup>−/−</sup> mice. OPN was mostly found in the adventitia and neointima, although some SMCs in the media were also strongly positive (Figure 4H and 4I). In WT mice, the OPN-positive area in the neointima equaled 18±15% (n=16; Figure 4H). Consistent with increased collagen deposition, the OPN positivity was significantly increased in the neointima of Tsp1<sup>−/−</sup> mice (32±20%; n=16; P<0.05 versus WT, Figure 4I). Western blots of vascular extracts confirmed that OPN expression was induced by the carotid artery ligation both in WT and in Tsp1<sup>−/−</sup> vessels (not shown), but was upregulated to higher levels in Tsp1<sup>−/−</sup> mice at day 28 (Figure 4J). Hence, Tsp1<sup>−/−</sup> neointimas have less cells, less contractile SMA-positive cells, more collagen, and accordingly, more OPN, a marker for collagen-producing cells.

Discussion

This study provides functional evidence for a role of TSP1 in activation, proliferation, and migration of SMCs in the vessel wall after vascular injury. The key findings of this study are: (1) loss of TSP1 delays SMC activation; (2) loss of TSP1 reduces SMC proliferation and MMP2 expression; (3) loss of

![Figure 4. Neointima composition in WT and Tsp1<sup>−/−</sup> mice. Representative light microscopy photos of SMA-stained (A, B), SR-stained (D, E), H&E-stained (F, G) and OPN-stained (H, I) WT or Tsp1<sup>−/−</sup> carotid arteries 28 days after ligation, as indicated. In the Immunoblots for SMA (C) and the 25 kDa OPN isoform (J), respectively 0.5 μg and 10 μg of protein from vascular extracts were loaded. Diagrams composed of the proportional SMA-positive, SR-positive, and OPN-positive areas in neointimas (58%, 28%, and 19%=105% in WT; respectively 22%, 54%, and 32%=108% in Tsp1<sup>−/−</sup>), readjusted to 100% for each genotype (K, L). Bar=50 μm.]
TSP1 impairs SMC migration resulting in smaller neointimas and thicker media 4 weeks after carotid artery ligation; (4) loss of TSP1 promotes collagen and osteopontin production in the late phase after ligation, altering the SMC phenotype.

Ligation of the carotid artery causes medial cell death the first 2 days. Cell death results in the concomitant release of growth factors and is followed by medial SMC activation and proliferation.\(^{33,30}\) This response was strongly attenuated in \(Tsp1^{-/-}\) media. Indeed, whereas WT medial SMC numbers rose between day 3 and 7 after ligation, \(Tsp1^{-/-}\) medial SMC densities hardly changed. These differences could be attributed to a 2-fold reduction in the proliferation rate of \(Tsp1^{-/-}\)-medial SMCs compared with WT, throughout the early recovery phase, possibly in conjunction with enhanced apoptosis of medial SMCs. Furthermore, the SMC response to the ligation between day 3 and 7, was delayed, noticeable from retarded medial thickening in \(Tsp1^{-/-}\) mice and from retarded reduction of SMA expression, characteristic of noncontractile SMC phenotype. The reduced MMP2 levels in \(Tsp1^{-/-}\) mice at day 3 and 7 after ligation, are in agreement with defective SMC migration, because MMP2 expression is associated with SMC migration. Indeed, \(MMP2^{-/-}\) mice develop significantly less neointima after carotid artery ligation than WT controls, because of defective degradation of ECM proteins surrounding migrating SMCs.\(^{37}\) Furthermore, in vitro, TSP1 upregulates MMP2 by transcriptional and nontranscriptional mechanisms, and MMP2 activation is relevant for TSP1-induced vascular SMC migration.\(^{28}\) Our findings therefore indicate that vascular TSP1 participates in early SMC activation after vessel occlusion, affecting morphological features of medial SMC and triggering SMC proliferation and migration.

In WT mice, the early proliferation and migration of medial SMCs after carotid artery ligation are followed by a reduction of SMC numbers in the media and the development of a neointima, as SMCs migrate into the lumen.\(^{33}\) In contrast, in \(Tsp1^{-/-}\) mice, as SMC numbers in the media did not change beyond day 3, \(Tsp1^{-/-}\) vessels persisted with a thick media and a 2-fold smaller neointima at day 28. Detailed morphological analysis of the outward remodeling, accompanying neointima formation in WT and \(Tsp1^{-/-}\) mice, revealed the same relationship in both genotypes between the extent of neointima formation and vascular enlargement, as measured via the external elastic lamina area (EEL). This analysis underscores that the smaller remodeling in \(Tsp1^{-/-}\) mice is secondary to the defective smooth muscle cell phenotype and does not stem from a more direct role for TSP1 in vascular remodeling. The less neointima, thicker media, and attenuated vessel enlargement in \(Tsp1^{-/-}\) vessels resulted in comparable luminal patency in WT and in \(Tsp1^{-/-}\) vessels.

Nevertheless, the deficiency of TSP1 modified the SMC phenotype in the media and in the neointima, and altered the neointima composition into a more fibrotic phenotype. In WT mice, mature neointimal lesions are composed mostly of SMA-positive cells.\(^{33}\) At completion of the neointimal proliferation, these cells return to a resting and contractile state, characterized by SMA expression.\(^{33}\) In the present study, we found that more than half of the WT neointimal area at day 28 consisted of SMA-positive cells, the remainder being covered by either collagen or osteopontin positive areas (marker for collagen-producing SMCs). In contrast, collagen was the main component of \(Tsp1^{-/-}\) neointima (more than half of the area) and the amount of osteopontin was significantly increased, with only a small area consisting of SMA-positive cells (Figure 4K and 4L). These findings indicate that in the absence of TSP1, SMCs differentiate into a more fibrotic collagen-producing SMC type. In view of the similar relationship between EEL and neointima in WT and \(Tsp1^{-/-}\) mice, the enhanced fibrosis is not greatly affecting eutrophic outward remodeling.

Because our data suggest that TSP1 modulates SMC function in the early phase (3 to 7 days) after ligation, knowledge of the SMC receptor(s) involved is highly relevant. Both CD47\(^{40}\) and \(\beta3^{+/-}\) mice show reduced neointima formation after carotid artery ligation, primarily because of defective SMC proliferation and migration, respectively. Several recent studies have identified CD47 as the major TSP1 receptor on SMCs, mediating the activity of TSP1 at physiological concentrations. Hence, whereas the neutralization of either CD36 or CD47 is sufficient to inhibit NO-stimulated vascular cell responses, only CD47 is necessary for this activity.\(^{41}\) Likewise, CD47 is the necessary receptor in vivo to explain the limiting NO-mediated vascular smooth muscle relaxation and tissue survival after ischemic injury in skin flaps and hindlimbs.\(^{3}\) These findings are in agreement with defective SMC proliferation and neointima formation in models of carotid artery ligation in CD47\(^{-/-}\) and \(Tsp1^{-/-}\) mice. Taken together, these results strongly suggest that CD47 is the receptor responsible for the presently described SMC phenotype.

In agreement with many in vitro studies, the present study points to a stimulatory effect for TSP1 on SMCs. Yet, TSP1 can behave as a strong SMC inhibitor, eg, antagonizing nitric oxide stimulated vascular SMC responses, in vitro and in vivo\(^{3}\) and endothelial cell activation (ECs).\(^{44}\) Although TSP1 is generally a positive regulator for SMCs and an inhibitor for ECs, these opposite roles are not surprising taking in consideration the multidomain structure of TSP1. Furthermore, the function of TSP1 in a specific environment is determined by receptor availability, molecular orientation, state of cell activation, and TSP1 concentration. Therefore, considering both the high TSP1 expression in WT neointima and the persistent noncontractile SMC phenotype in the neointima of \(Tsp1^{-/-}\) mice, TSP1 may actively contribute to arresting SMC proliferation in the matured neointima, ie, facilitate their return to the dedifferented contractile state.

In summary, we provided evidence for a role for TSP1 in SMC activation after mild vascular injury. TSP1 facilitates SMC proliferation and migration after injury and the development of a neointima. In the absence of TSP1, proliferation and migration of SMCs are reduced and a more fibrotic neointima develops.

**Acknowledgments**

We thank M. Van Hul for the help with the zymograms, L. Frederix and S. Tercavelers for excellent technical assistance, and Prof J. Lawler and Prof Em J. Vermelen for critically reading the manuscript.
**Sources of Funding**

This work was supported by K.U. Leuven grant GOA/2004/09 and by FWO Vlaanderen (project no. G.0569.05). The CMVB is supported by the “Excellente financiering KULeuven”. M.T. is a research fellow of the Belgian IWT.

**Disclosures**

None.

**References**

Thrombospondin-1 Activates Medial Smooth Muscle Cells and Triggers Neointima Formation Upon Mouse Carotid Artery Ligation
Rute Moura, Marc Tjwa, Petra Vandervoort, Katrien Cludts and Marc F. Hoylaerts

Arterioscler Thromb Vasc Biol. 2007;27:2163-2169; originally published online August 30, 2007;
doi: 10.1161/ATVBAHA.107.151282

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2007 American Heart Association, Inc. All rights reserved.
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:
http://atvb.ahajournals.org/content/27/10/2163

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2007/09/20/ATVBAHA.107.151282.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
Thrombospondin-1 activates medial smooth muscle cells and triggers neointima formation upon mouse carotid artery ligation

Rute Moura¹, Marc Tjwa², Petra Vandervoort¹, Katrien Cludts¹, Marc F Hoylaerts¹

¹Center for Molecular and Vascular Biology, University of Leuven, Belgium; ²Center for Transgene Technology and Gene Therapy, University of Leuven, Belgium;
METHODS

Mice and model of carotid artery ligation

Briefly, mice were anesthetized with ketamine/xylazine (80/5 mg/kg), the left common carotid artery was dissected free of connective tissue and ligated proximally to the bifurcation with 5/0 silk thread. At specific time points after surgery, the animals were anesthetized with Nembutal (60 mg/kg) and transcardially perfused with 1% paraformaldehyde under physiological pressure.

Histological and morphometric analysis

Tissue preparation: Postfixed carotid arteries were rinsed with Phosphate-Buffered Saline, immersed in 70% ethanol, dehydrated in increasing concentrations of ethanol, cleared in xylol and embedded in paraffin. Paraffin serial sections of 7µm were prepared using a HM360 microtome (Microm, Walldorf, Germany), starting 5 mm proximal to the ligation site towards the ligated area. To avoid crush injury artefacts, an area of 1 mm close to the ligation site was discarded. The distal area between 1 mm and 3 mm of the ligation site was sectioned and the 250-300 sections divided over 10 consecutive glass slides. Hence, each slide contained approximately 25 sections, adjacent sections being 70 µm apart in the native carotid artery.

Histochemical stainings: paraffin sections were deparaffinized in xylol and rehydrated in decreasing concentrations of ethanol. Staining with hematoxylin and eosin (H&E) was performed on the first section of every series. For collagen measurement, sections were stained with the sulphonated azo-dye Sirius red. Sirius red staining was also used to study collagen organization by polarization microscopy.

Immunohistochemical stainings: staining conditions were adjusted depending on the staining. In general, antigen retrieval was performed by incubation for 20 minutes in Dako Target Retrieval Solution (Dako, Carpinteria, USA) at 90°C. Endogenous peroxidase activity was blocked by incubation for 20 minutes in methanol 0.3% H₂O₂, followed by incubation with appropriate serum to mask non-specific binding sites. Sections were then incubated overnight with antibodies against SMC actin (1/500 Dako SMA Mo851), CD45 (1/100 BD 553076), MAC3 (1/50 Pharmingen Rat anti-MAC3), OPN (4µg/ml Santa Cruz Biotechnology, sc-10593) and TSP1 (5 µg/ml home-made Rabbit-anti-human TSP1 biotinylated, pre-adsorbed with liver extract from Tsp1−/− mice, which procedure strongly reduced interfering TSP2 signals). With
SMA, CD45, MAC3 and OPN, appropriate peroxidase (PA)-labeled secondary antibodies were used and peroxidase activity was visualized using 0.06% 3,3'-diaminobenzidine (DAB)/0.01% H₂O₂.

**Microscopic analysis:** 6-8 mice per group and the 4 most representative, i.e. maximally response, adjacent sections per mouse were analyzed on each glass, covering a longitudinal carotid artery distance of approximately 280 µm, resulting in N=14-36 measurements, statistically treated as independent. Images were acquired with the Zeiss AxioVision software and a Zeiss AxioPlan 2 imaging microscope equipped with a Zeiss AxioCam HrC camera. Analysis of acquired images was performed using the Zeiss KS300 software and quantification of stained areas was achieved by automatic detection of color through color segmentation of the images.

**RESULTS:**

**Vessel inflammation in WT and Tsp1⁻/⁻ carotid arteries after ligation**

Table I shows that the early inflammatory response in both genotypes is identical. Both 3 and 7 days after ligation, the numbers of granulocytes and macrophages, invading the media and adventitia were identical. These findings indicate that the initial adhesion and invasion of inflammatory cells to hypoxia-activated endothelium did not depend on TSP1.

**Carotid artery morphometry, neointima development, Geometric remodeling and vascular patency after carotid artery ligation**

Morphometric analysis of carotid artery cross-sections of WT and Tsp1⁻/⁻ mice showed vascular remodeling in both genotypes (Fig. I). The external elastic lamina area (EEL) and medial area increased in WT but not in Tsp1⁻/⁻ mice (Fig. Ia,b). Medial area index (medial area/ EEL area), a parameter of medial thickening, was increased in WT at day 3 and 7, and in Tsp1⁻/⁻ mice at day 7 and 28 (Fig. Ic).

Arterial remodeling in both genotypes was studied by plotting the EEL at day 28 as a function of the neointima formed. This analysis revealed a linear relationship in WT cross-sections and Tsp1⁻/⁻ cross-sections, with comparable slopes and intersections in both
genotypes (Fig. Ie,f). Thus, the relative outward eutrophic geometric remodeling in WT and $Tsp1^{-/-}$ mice was identical, its extent in $Tsp1^{-/-}$ mice being determined by the smaller neointima, in these mice (Fig. Id), ultimately leading to a comparable luminal cross-sectional area, on day 28, in WT and $Tsp1^{-/-}$ mice (Fig. Id).

**MMP2 and MMP9 activation, after ligation**

Fig. II shows the position of MMP9, Pro-MMP2 and active MMP2 in a developed zymogram, as a function of time and mouse genotype, The MMP activities present in cell extracts of HT10890 cells, confirmed that the position of the indicated MMP bands was correct. Fig. II shows rapid expression of MMP2 in WT but not in $Tsp1^{-/-}$ mouse carotid arteries.

**Loss of contractile SMC phenotype and collagen turnover in ligated carotid arteries**

We used the absence of SMA to identify the phenotype switch from contractile to synthetic. At baseline, medial SMA staining was comparable between genotypes (Fig. III). In WT mice, medial SMA staining was strongly reduced 3 days after ligation; this happened in $Tsp1^{-/-}$ mice only at day 7 (Fig. III). These results suggest that loss of the contractile SMC phenotype in $Tsp1^{-/-}$ mice was delayed. Consistently, SMA staining had returned to normal levels in WT cross-sections at day 28, but remained attenuated in $Tsp1^{-/-}$ sections.

The collagen deposition in media and adventitia was analyzed morphometrically in Sirius Red stained cross-sections. Fig. IVa shows that the area covered with collagen increases in the media of $Tsp1^{-/-}$ mouse carotid arteries at day 28, and is more elevated at this time point that in WT media. Fig. IVb shows that collagen deposition is regulated differentially by SMCs only, since no genotypic difference was observed for Sirius Red staining in the adventitia.

To assess differences in collagen fiber organization, SR-stained sections were also analyzed under polarized light\(^1\), where thick and tightly packed collagen fibers appear red-orange, while thin and loosely assembled fibers appear green-yellow. No obvious differences in the collagen organization were found between genotypes. Collagen fibers were typically red-orange under polarized light in both genotypes, with the amount of total fibers simply being more abundant in sections of $Tsp1^{-/-}$ mice (Fig. IVc-f). Thus, in the absence of TSP1, deposition of normally organized collagen fibers is increased in the media.
REFERENCE:


FIGURE LEGENDS:

Figure I: Morphological parameters in WT and Tsp1<sup>−/−</sup> carotid arteries after ligation. EEL area (a), Medial area (b), and Medial area index (c) as a function of time for WT (black bars) and Tsp1<sup>−/−</sup> carotid arteries (white bars). Neointima development, as a percentage of the internal elastic lamina area (IEL), and vascular patency, as luminal area, 4 weeks after ligation (d); relationship between EEL and neointima for WT (n = 25) and Tsp1<sup>−/−</sup> cross-sections (n = 25), as indicated (e,f).

Figure II: Gelatinase activity in protein extracts from carotid arteries. Vessels were harvested at the indicated time-points and analysed by zymography. Five micrograms of protein from vascular extracts were loaded per lane, and cell extract of HT1080 cells served as an internal reference for identification of MMP2 and 9 activity bands.

Figure III: SMC phenotype analysis via SMA staining. Representative light microscopy photos of SMA-stained carotid arteries from WT and Tsp1<sup>−/−</sup> mice, as indicated, at day 0 (a,b), 3 (c,d), 7 (e,f) and 28 (g,h). Note the loss of SMA staining of the media at day 3 and 7 in WT arteries and at day 7 and 28 in Tsp1<sup>−/−</sup> arteries. Bar: 50 µm.

Figure IV: Collagen staining in carotid arteries 4 weeks after ligation. Sirius red positive area in the media (a) and adventitia (b) of WT and Tsp1<sup>−/−</sup> mice as a function of time; Bright field microscope images of SR-stained medias of WT and Tsp1<sup>−/−</sup> mice as indicated (c,d) and polarization microscope images of the same areas (e,f); (A: adventitia; M: media; NI: neointima); arrows indicate IEL and EEL; Bar: 10 µm.
Table I: Total inflammatory cell numbers infiltrated in the intima, media and adventitia, at day 3 and day 7 after ligation.

*Quantification was done on carotid artery sections stained for CD45 (leukocytes) or MAC3 (macrophages).

<table>
<thead>
<tr>
<th></th>
<th>Average cell count*</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
<td>Tsp1−/−</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Media &amp; Intima</td>
<td>Adventitia</td>
<td>Media &amp; Intima</td>
<td>Adventitia</td>
<td></td>
</tr>
<tr>
<td>CD45</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 3</td>
<td>19,25</td>
<td>16</td>
<td>16,3</td>
<td>15,75</td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td>3,3</td>
<td>6</td>
<td>5,7</td>
<td>3,5</td>
<td></td>
</tr>
<tr>
<td>Day 7</td>
<td>23,8</td>
<td>31,8</td>
<td>21,27</td>
<td>27,3</td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td>12,2</td>
<td>9,9</td>
<td>8,1</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>MAC3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 3</td>
<td>5</td>
<td>9,75</td>
<td>6,5</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td>1,4</td>
<td>5,1</td>
<td>3,3</td>
<td>2,4</td>
<td></td>
</tr>
<tr>
<td>Day 7</td>
<td>3,6</td>
<td>7,12</td>
<td>5</td>
<td>7,56</td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td>2,3</td>
<td>1,6</td>
<td>1,6</td>
<td>2,9</td>
<td></td>
</tr>
</tbody>
</table>

N=16-20
Figure I
WT \quad Tsp1^{-/-}

---

MMP9

Pro-MMP2

Activated MMP2

Days after ligation

0 3 7 28 0 3 7 28

Figure II
Figure III
Figure IV

a, b: Bar graphs showing the percentage of Sirius Red stained area in the adventitia for Day 0, Day 3, and Day 28. The graphs indicate statistical significance with P<0.0001 and P<0.002.

c, d: Images of WT and Tsp1^-/- vessels stained with Sirius Red. The images show the area of interest (NI), muscle (M), and adventitia (A).

e, f: Additional images with similar staining and labeling.