Published in final edited form as:

J Mol Cell Cardiol. 2007 August ; 43(2): 210–214.

Vascular Response to Intra-arterial Injury in the Thrombospondin-1 Null Mouse

Faisal Budhani^{*}, Katherine A. Leonard^{*}, Andreas Bergdahl^{*}, Jimin Gao^{†,b}, Jack Lawler[‡], and Elaine C. Davis^{*,a}

^{*} Department of Anatomy and Cell Biology, McGill University, Montreal, Quebec, Canada, H3A 2B2

[†] Department of Cell Biology, University of Texas Southwestern Medical Center, Dallas, TX, 75390

[‡] Department of Pathology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts, USA, 02215

Abstract

Thrombospondin-1 (TSP-1) is a multifunctional, extracellular matrix protein that has been implicated in the regulation of smooth muscle cell proliferation, migration and differentiation during vascular development and injury. Vascular injury in wildtype and TSP-1 null mice was carried out by insertion of a straight spring guidewire into the femoral artery via a muscular arterial branch. Blood flow was restored after the muscular branch was ligated. The injury completely denuded the endothelium and caused medial distension of the vessel in a manner similar to coronary artery balloon-angioplasty. After 28 days, wildtype arteries showed consistent neointima formation with smooth muscle cell hyperplasia. Injured arteries from TSP-1 null mice showed similar neointimal lesions with no significant difference in the extent of neointima formation. Unexpectedly, a high incidence of thrombus formation was observed in the TSP-1 null vessels in a region close to the entry point of the guidewire into the femoral artery. Thrombus was never observed in the injured wildtype vessels. These results provide *in vivo* evidence that the extent of smooth muscle cell proliferation and neointima formation following endothelial denuding injury is not affected by the absence of TSP-1. Furthermore, our results provide novel evidence for the involvement of TSP-1 in controlling thrombus growth following intra-arterial injury in areas of predicted high turbulent flow.

Keywords

arterial injury; knockout mouse; neointima; thrombosis; thrombospondin-1

1. Introduction

Thrombospondin-1 (TSP-1) is a matricellular glycoprotein that exists as a homotrimer of approximately 450 kDa. It is synthesized by several cell types, including fibroblasts, endothelial cells (ECs) and smooth muscle cells (SMCs), and has been reported to be involved in numerous functions such as cell differentiation, adhesion, migration, proliferation and survival (for review see [1]). With such a wide range of functions, and the fact that TSP-1 is highly expressed in many developing tissues [2], investigators believed that deletion of the

^a Address for correspondence: Dr. Elaine C. Davis, Department of Anatomy and Cell Biology, McGill University, 3640 University Street, Montreal, Quebec, Canada H3A 2B2, phone: 514-398-5893, fax: 514-398-5047, E-mail: elaine.davis@mcgill.ca

^b Current address: Dr. Jimin Gao, Institute of Biotherapy, Wenzhou Medical College, Wenzhou, Zhejiang, P. R. China 325035

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

TSP-1 gene (*Tsp1*) might result in an embryonic lethal phenotype. In contrast, *Tsp1*^{-/-} mice were viable and fertile with only subtle and variable defects [3]. Despite *in vitro* experiments showing that TSP-1 can stimulate SMC proliferation and that this property can be blocked by a TSP-1 antibody [4,5], the vasculature of the *Tsp1*^{-/-} mice appeared normal, thus raising question as to the *in vivo* significance of TSP-1 in SMC function. In the present study, the *in vivo* function of TSP-1 was investigated using a vascular injury model performed on wildtype (WT) and *Tsp1*^{-/-} mice. The model, which involves insertion of a straight spring guidewire into the femoral artery, causes reproducible neointimal hyperplasia as a consequence of mechanical distension and endothelium denudation [6], thereby resembling human coronary artery balloon-angioplasty.

2. Materials and methods

2.1. Animals

WT and *Tsp1*^{-/-} C57BL/6 male mice [3], generated from *Tsp1*^{+/-} x *Tsp1*^{+/-} matings, were used between 3 and 4 months of age. Surgeries on the mice of the two genotypes were conducted in random order.

2.2. Femoral Artery Injury

A mouse femoral artery injury model, approved by the Animal Care Committee at McGill University, was conducted as previously described [6] (see figure 2 for details). Materials used were 50 mg/kg Somnotol (MTC Pharmaceuticals, Cambridge, ON) diluted in 0.9% NaCl for anesthesia, 1% lidocaine hydrochloride (Sigma, St Louis, MO) for topical vessel dilation and a straight spring guidewire (0.38 mm diameter, No. C-SF-15-15, Cook, Bloomington, IN) for intra-arterial injury.

2.3. Preparation of Light (LM) and Electron Microscope (EM) Sections

Twenty-eight days following femoral artery injury, mice were anesthetized, perfusion fixed and the injured and contralateral control femoral arteries prepared for Epon embedding. For embedding, each vessel was cut into two segments and oriented such that sections would be obtained at 6 mm and 2 mm proximal to the entry point of the guidewire (Seg1 and Seg2, see figure 2). Semi-thin sections (0.5 µm) were stained with toluidine blue for LM and thin sections (60 nm) were stained with methanolic uranyl acetate and lead citrate for EM.

2.4. Staining for Elastin and von Willebrand factor (vWF)

One hour following injury, mice were anesthetized and perfused with saline. The injured and contralateral femoral arteries were removed and immersion fixed in 4% paraformaldehyde before paraffin embedding. For elastin staining, a modified Hart's stain was used. For vWF localization, a rabbit anti-human vWF polyclonal antibody at 1:200 (Chemicon, Temecula, CA) and a ImmunoCruz™ Staining Kit (Santa Cruz Biotechnology, Santa Cruz, CA) were used. Sections were counterstained with methylene blue.

2.5. Section Analysis and Statistics

To quantify neointima formation in WT (Seg1 and Seg2) and *Tsp1*^{-/-} (Seg1) vessels, intimal and medial areas were determined and expressed as intima/media ratios. Statistical analysis was performed using an unpaired, two-tailed Student's *t*-test. For the occurrence of neointimal formation in segment 2, a two-tailed Fisher's Exact Test was used. Results are reported as means ± S.D. with a significance level of *p* < 0.05.

3. Results

Prior to conducting the vascular injury studies, femoral arteries of WT and *Tsp1*^{-/-} mice were investigated by EM to ensure that the absence of TSP-1 did not alter the general morphology (Figs. 1A, B). To confirm vascular distension and endothelial denudation, as previously reported for this model [6,7], elastin and vWF were localized in vessels from WT and *Tsp1*^{-/-} mice 1 hour after surgery. The control (contralateral, sham operated) vessel showed a continuous internal elastic lamina (IEL) with undulations indicating elastic recoil and an intact EC layer as indicated by positive vWF immunostaining (Figs. 1C, D). In contrast, the injured vessel showed considerable distension and the absence of vWF immunostaining (Figs. 1E, F). Acute thrombus was never observed with either genotype. EM analysis confirmed the absence of ECs, disruption of the SMC layer due to distension, and the presence of platelets adhered to the exposed IEL and subendothelial matrix (Figs. 1G, H).

Two regions of the WT and *Tsp1*^{-/-} femoral arteries were analyzed by LM 28 days following vascular injury (Fig. 2, Seg1 and Seg2). Analysis of Seg1 in both WT and *Tsp1*^{-/-} vessels showed consistent neointima development resulting in reduction of lumen diameter (Figs. 2A, B). No significant difference in lumen diameter between the two genotypes was observed ($p = 0.655$). A similar neointimal response was also observed in Seg2 of WT vessels (Fig. 2C). In contrast, Seg2 in the *Tsp1*^{-/-} mice showed neointimal lesions in only 2 out of 15 *Tsp1*^{-/-} vessels examined, with all others showing some degree of thrombus formation (Figs. 2D, E). Thrombus formation was never observed in the WT vessels. Quantification of the extent of neointima formation for WT (Seg1 and Seg2) and *Tsp1*^{-/-} (Seg1) vessels showed no significant difference between any of the segments (I/M ratios: WT Seg1 = 1.50 ± 0.30 ; WT Seg2 = 1.85 ± 0.22 ; *Tsp1*^{-/-} Seg1 = 1.85 ± 0.31) (Fig. 2F).

4. Discussion

The present study provides *in vivo* evidence that neointima formation as a response to endothelium denuding vascular injury is not significantly altered by the absence of TSP-1. Others, however, have reported that intra-arterial administration of a TSP-1 antibody can reduce neointima formation after endothelium denudation in rat carotid arteries [8], and that TSP-1 antibodies can block SMC proliferation *in vitro* [4]. Recently, Isenberg et al. [9] showed that the inhibitory activity of TSP-1 antibodies on SMC proliferation was due to cross-linking of endogenous TSP-1 bound to cell surface receptors rather than the antibody acting as an antagonist to TSP-1 stimulation. Using *Tsp1*^{-/-} SMCs, these authors reevaluated the role of endogenous TSP-1 and found it to be non-essential for SMC proliferation, but permissive for the chemotaxis response of SMCs to platelet-derived growth factor (PDGF) [9]. These recent data support our finding that no difference in neointima formation, and thus SMC migration and proliferation, was seen in the absence of TSP-1 following intra-arterial injury. Although the *Tsp1*^{-/-} SMCs may be defective in their response to PDGF, other mitogens such as insulin-like growth factor, which is markedly upregulated after arterial wall injury [10], would still function as a powerful mitogen for the SMCs irrespective of the presence of endogenous TSP-1 [9].

An unexpected finding in this study was that thrombus formation was observed exclusively in the *Tsp1*^{-/-} femoral artery in the region closest to guidewire entry. This suggests the interesting possibility that TSP-1 plays a contributing role, either directly or indirectly, in the control of thrombus growth following vascular injury. In both WT and *Tsp1*^{-/-} mice, the only difference between segments 1 and 2 is anatomic location; segment 1 being unbranched and subjected to laminar flow, whereas segment 2, being subjected to altered blood-flow dynamics due to its close proximity to the small, blind-ended pocket that would remain after ligation of the muscular branch. Turbulence is a known contributing factor to intravascular thrombus

formation [11,12], however, this alone was not sufficient to induced thrombus formation since WT vessels never showed the presence of thrombus. *Tsp1*^{-/-} platelets release larger vWF multimers than WT platelets and show increased collagen- and vWF-mediated aggregation [13]. Thus, the random, agitated motion of blood-flow in the vessel near the muscular branch bifurcation, together with the propensity of the TSP-1 null platelets to aggregate, may have lead to thrombus formation in the injured *Tsp1*^{-/-} vessel.

Recently, Bonnefoy and colleagues [14] reported that occlusive thrombi formed twice as quickly in arterioles of WT compared to *Tsp1*^{-/-} mice using a photochemical injury model. The vessels examined, however, were small arterioles located in the vascular bed of the cecum; distinctly different than the femoral artery. Additionally, although the photochemical reaction does cause endothelial cells to contract and detach from the vessel wall [15], no mechanical distension or subendothelial matrix disruption occurs. Consistent with the two models being very different is the fact that complete thrombi formed in approximately 12 minutes in WT cecal arterioles following photochemical injury [14], whereas, in our study, WT vessels showed only a layer of adherent platelets one hour after guidewire removal (Figs. 1G, H and [7]). Thus, the two models and data obtained can not be directly compared.

5. Conclusions

The present study provides *in vivo* evidence that TSP-1 is not a significant contributing factor in SMC migration and proliferation during neointima formation as a response to a vascular injury involving endothelium denudation and mechanical distension. The novel finding that thrombus formation occurs exclusively in the *Tsp1*^{-/-} femoral artery in response injury and predicted aberrant turbulent flow suggests the possibility of an important interplay between flow dynamics, platelet activation and TSP-1 function.

Acknowledgements

The authors thank Dr. Masataka Sata for his advice on the femoral artery vascular injury model, Ms. Jeannie Mui for technical assistance with tissue sectioning and Jiwon Choi for statistical analysis. This work was supported by National Institutes of Health grant HL68003 (JL) and Canadian Institutes of Health Research grant MOP-57663 (ECD). ECD is a Canada Research Chair.

References

1. Lawler J. The functions of thrombospondin-1 and -2. *Curr Opin Cell Biol* 2000;12:634–40. [PubMed: 10978901]
2. Iruela-Arispe ML, Liska DJ, Saga EH, Bornstein P. Differential expression of thrombospondin 1, 2 and 3 during murine development. *Dev Dyn* 1993;197:40–56. [PubMed: 8400410]
3. Lawler J, Sunday M, Thibert V, Duquette M, George EL, Rayburn H, et al. Thrombospondin-1 is required for normal murine pulmonary homeostasis and its absence causes pneumonia. *J Clin Invest* 1998;101:982–92. [PubMed: 9486968]
4. Majack RA, Goodman LV, Dixit VM. Cell surface thrombospondin is functionally essential for vascular smooth muscle cell proliferation. *J Cell Biol* 1988;106:415–22. [PubMed: 2448314]
5. Majack RA, Cook SC, Bornstein P. Control of smooth muscle cell growth by components of the extracellular matrix: autocrine role for thrombospondin. *Proc Natl Acad Sci USA* 1986;83:9050–4. [PubMed: 3491367]
6. Sata M, Maejima Y, Adachi F, Fukino K, Saiura A, Sugiura S, et al. A mouse model of vascular injury that induces onset of medial cell apoptosis followed by reproducible neointimal hyperplasia. *J Mol Cell Cardiol* 2000;32:2097–104. [PubMed: 11040113]
7. Roque M, Fallon JT, Badimon JJ, Zhang WX, Taubman MB, Reis ED. Mouse model of femoral artery denudation injury associated with the rapid accumulation of adhesion molecules on the luminal surface and recruitment of neutrophils. *Arterioscler Thromb Vasc Biol* 2000;20:335–42. [PubMed: 10669628]

8. Chen D, Asahara T, Krasinski K, Witzenbichler B, Yang J, Magner M, et al. Antibody blockade of thrombospondin accelerates reendothelialization and reduces neointima formation in balloon-injured rat carotid artery. *Circulation* 1999;100:849–54. [PubMed: 10458722]
9. Isenberg JS, Calzada MJ, Zhou L, Guo N, Lawler J, Wang XQ, et al. Endogenous thrombospondin-1 is not necessary for proliferation but is permissive for vascular smooth muscle cell responses to platelet-derived growth factor. *Matrix Biol* 2005;24:110–23. [PubMed: 15890262]
10. Khorsandi MJ, Fagin JA, Giannella-Neto D, Forrester JS, Cercek B. Regulation of insulin-like growth factor-I and its receptor in rat aorta after balloon denudation. Evidence for local bioactivity. *J Clin Invest* 1992;90:1926–31. [PubMed: 1430215]
11. Smith RL, Blick EF, Coalson J, Stein PD. Thrombus production by turbulence. *J Appl Physiol* 1972;32:261–4. [PubMed: 5007881]
12. Stein PD, Sabbah HN. Measured turbulence and its effect on thrombus formation. *Circ Res* 1974;35:608–14. [PubMed: 4278187]
13. Pimanda JE, Ganderton T, Maekawa A, Yap CL, Lawler J, Kershaw G, et al. Role of thrombospondin-1 in control of von Willebrand factor multimer size in mice. *J Biol Chem* 2004;279:21439–48. [PubMed: 14981081]
14. Bonnefoy A, Daenens K, Feys HB, De Vos R, Vandervoort P, Vermeylen J, et al. Thrombospondin-1 controls vascular platelet recruitment and thrombus adherence in mice by protecting (sub)endothelial VWF from cleavage by ADAMTS13. *Blood* 2006;107:955–64. [PubMed: 16204318]
15. Saniabadi AR, Umemura K, Matsumoto N, Sakuma S, Nakashima M. Vessel wall injury and arterial thrombosis induced by a photochemical reaction. *Thromb Haemost* 1995;73:868–72. [PubMed: 7482418]

Figure 1. Ultrastructure of the femoral artery and effect of guidewire insertion

EM of the femoral artery in WT (A) and *Tsp1*^{-/-} (B) mice shows no ultrastructural differences due to the absence of TSP-1. Prior to guidewire insertion, the artery shows an undulating IEL due to normal elastic recoil (C) and an intact EC layer as shown by vWF immunostaining (arrow) (D). One hour after insertion and removal of the guidewire, both distension (E) and EC denudation based on the lack of vWF immunostaining (arrow) (F) can be seen. By EM, the medial SMCs appear disrupted and platelets are observed in the lumen of the vessel (L), adjacent to the IEL (G). At higher magnification (H), it is clear that the IEL is directly exposed to the platelets (P). Bars = 2 μ m (A, B, G); 50 μ m (C-F); 1 μ m (H).

Figure 2. Vascular response of the femoral artery 28 days following guidewire injury
At the left is a line drawing representing the left femoral artery at the completion of the surgery. Before surgical intervention, sutures are placed around proximal and distal portions of the femoral artery (stars) and pulled forward to restrict blood flow. A lateral muscular branch is then ligated (Lig1) and an incision made just proximal to the ligature for insertion of the guidewire. The guidewire is thread proximally into the femoral artery towards the iliac artery (~12 mm), left in place for 2 minutes, then withdrawn and the muscular branch ligated (Lig2). The sutures around the femoral artery are released and blood-flow is restored. For analysis, two segments of the femoral artery were studied; approximately 6 mm (Seg1) and 2 mm (Seg2) from the bifurcation of the muscular branch. Analysis of Seg1 in WT (A) and *Tsp1*^{-/-} mice (B) showed only neointima formation (NI). Seg2 from WT mice (C) showed a similar neointima lesion. In contrast, Seg2 from *Tsp1*^{-/-} mice (D) showed a high incidence of thrombus formation. Neointima formation was only observed in 2 out of 15 *Tsp1*^{-/-} mice with the remaining *Tsp1*^{-/-} mice showing partial to complete occlusive thrombus (E). No significant difference in intima/media ratio was found between WT Seg1, WT Seg2 or *Tsp1*^{-/-} Seg1 (F). Arrowheads in A and B show the IEL. Bar = 50 μm.