Biochemical and Biophysical Research Communications xxx (2015) 1–6

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Thrombospondin-1, -2 and -5 have differential effects on vascular smooth muscle cell physiology

Alex Helkin [a, b], Kristopher G. Maier [a, b, 1], Vivian Gahtan [a, b, *, 1]

[a] SUNY Upstate Medical University, Division of Vascular Surgery and Endovascular Services, Syracuse, NY, USA
[b] Department of Veterans Affairs VA Healthcare Network Upstate New York at Syracuse, Syracuse, NY, USA

ARTICLE INFO

Article history:
Received 1 July 2015
Accepted 8 July 2015
Available online xxx

Keywords:
Thrombospondins
Gene expression profiling
Vascular smooth muscles
Cartilage oligomeric matrix protein
Cell migration
Cellular proliferation

ABSTRACT

Introduction: The thrombospondins (TSPs) are matricellular proteins that exert multifunctional effects by binding cytokines, cell-surface receptors and other proteins. TSPs play important roles in vascular pathobiology and are all expressed in arterial lesions. The differential effects of TSP-1, -2, and -5 represent a gap in knowledge in vascular smooth muscle cell (VSMC) physiology. Our objective is to determine if structural differences of the TSPs imparted different effects on VSMC functions critical to the formation of neointimal hyperplasia. We hypothesize that TSP-1 and -2 induce similar patterns of migration, proliferation and gene expression, while the effects of TSP-5 are different.

Methods: Human aortic VSMC chemotaxis was tested for TSP-2 and TSP-5 (1–40 μg/mL), and compared to TSP-1 and serum-free media (SFM) using a modified Boyden chamber. Next, VSMCs were exposed to TSP-1, TSP-2 or TSP-5 (0.2–40 μg/mL). Proliferation was assessed by MTS assay. Finally, VSMCs were exposed to TSP-1, TSP-2, TSP-5 or SFM for 3, 6 or 24 h. Quantitative real-time PCR was performed on 96 genes using a microfluidic card. Statistical analysis was performed by ANOVA or *t*-test, with $p < 0.05$ being significant.

Results: TSP-1, TSP-2 and TSP-5 at 20 μg/mL all induce chemotaxis 3.1 fold compared to serum-free media. TSP-1 and TSP-2 induced proliferation 53% and 54% respectively, whereas TSP-5 did not. In the gene analysis, overall, cardiovascular system development and function is the canonical pathway most influenced by TSP treatment, and includes multiple growth factors, cytokines and proteases implicated in cellular migration, proliferation, vasculogenesis, apoptosis and inflammation pathways.

Conclusions and relevance: The results of this study indicate TSP-1, -2, and -5 play active roles in VSMC physiology and gene expression. Similarly to TSP-1, VSMC chemotaxis to TSP-2 and -5 is dose-dependent. TSP-1 and -2 induces VSMC proliferation, but TSP-5 does not, likely due conservation of N-terminal domains in TSP-1 and -2. In addition, TSP-1, -2 and -5 significantly affect VSMC gene expression; however, little overlap exists in the specific genes altered. This study further delineates TSP-1, -2 and -5's contributions to processes related to VSMC physiology.

Published by Elsevier Inc.

1. Introduction

Despite advances in angioplasty and stenting technology, restenosis secondary to neointimal hyperplasia remains a critical component of failure of both open and percutaneous revascularization procedures. Neointimal hyperplasia is characterized by vascular smooth muscle cell (VSMC) migration and proliferation following endothelial cell (EC) injury and induction of inflammatory pathways. Signaling between ECs, leukocytes, VSMCs and platelets in response to vessel injury have been shown to induce phenotypic changes in VSMCs, ultimately resulting in proliferation and deposition of extracellular matrix (ECM) [1]. One example of the proteins expressed in this injury pattern includes thrombospondins (TSPs), a family of multifunctional matricellular glycoproteins. While these proteins do not contribute to the normal ECM structural architecture, they do function in cellular signaling

Abbreviations: EC, endothelial cell; SFM, serum-free media; TSP, thrombospondin; VSMC, vascular smooth muscle cell.

* Corresponding author. Upstate Medical University, 750 East Adams Street, Syracuse, NY 13210, USA.
E-mail address: gahtanv@upstate.edu (V. Gahtan).

[1] These authors contributed equally to this publication.

http://dx.doi.org/10.1016/j.bbrc.2015.07.044
0006-291X/Published by Elsevier Inc.

by interacting with other ECM proteins, such as cell surface receptors, proteases and cytokines [2].

The TSP family consists of 5 member proteins, which are divided into Groups A (TSP-1, TSP-2) and B (TSP-3, TSP-4, TSP-5), determined by the inclusion (Group A) or exclusion (Group B) of Type 1 repeats, domains found at the N-terminal side of the molecules [3]. TSP-1 is the most studied TSP, and is established as a potent anti-angiogenic protein [4] and an inducer of VSMC chemotaxis and proliferation [5]. Functions such as inducing EC apoptosis are experimentally attributed to CD36 binding of Type 1 repeats, while others, such as CD47 and integrin binding are attributed to the relatively conserved C-terminal domain [6]. Previous experiments indicate hyperglycemia, dyslipidemia, leptin, inflammatory mediators, such as TGFβ1, and statins drugs all regulate TSP-1 expression and function [7–10], implying TSP-1's importance to vascular pathologies such as atherosclerosis and intimal hyperplasia. While TSP-1 is well characterized in the literature, less is known about the effects of other TSPs on VSMCs and their contributions to vascular disease progression.

TSP-2 is a Group A TSP, closely resembling TSP-1 in structure. Some of TSP-2's functions are reported to mimic those of TSP-1, i.e. inhibiting angiogenesis, inducing EC apoptosis and participating in integrin-mediated signaling pathways [2]. However, some key differences distinguish TSP-1 from TSP-2. While TSP-1 and TSP-2 are both secreted by VSMCs, macrophages and fibroblasts, TSP-1 is stored in platelet α granules, and released in relatively large quantities following arterial injury, whereas TSP-2 is not. In one study, a wound healing assay, the release of TSP-2 from VSMCs was not detected until 3 days following injury, and peaked as far out as 10 days post-injury [11], whereas TSP-1 was detected early after injury, peaking at 3 days and subsiding by day 7. This finding suggests TSP-1 may function more as an acute phase reactant, while TSP-2 may be more important during the subsequent inflammation and remodeling phases.

TSP-5, also known as cartilage oligomeric matrix protein (COMP), is a Group B TSP notably different in structure compared to Group A TSPs. While the C-terminus of TSPs is relatively conserved, the N-terminus of TSP-5 lacks Type 1 repeats, and is notably truncated compared to other TSPs [12]. Previous studies have identified TSP-5 in normal rat aorta [13], human VSMCs [12] and the atherosclerotic plaques of ApoE−/− mice [14], implicating an importance to vascular pathobiology and a potential target for therapy; however, few experiments exploring TSP-5 and its relevance to arterial disease have been performed to date.

In the present study, we sought to identify the differences Group A and B TSPs impart on VSMC migration, proliferation, and gene expression. Specifically, we hypothesized that: (1) similarities in TSP-1 and TSP-2 structure would induce similar VSMC chemotaxis and proliferation, as well as similar alterations in pro-migratory and pro-inflammatory genes; and (2) structural differences of TSP-5 would induce VSMC chemotaxis, but not migration, and provide a distinct pattern of gene expression.

2. Methods

2.1. Materials

TSP-1 was obtained from Athens Research (Athens, GA). Recombinant TSP-2 and TSP-5 were obtained from R&D Systems (Minneapolis, MN). Smooth muscle cell growth medium was purchased from Cell Applications, Inc. (San Diego, CA, USA). Dulbecco's Modified Eagle Medium (DMEM), used as serum-free media (SFM), trypsin and trypsin neutralizing solution were purchased from Lonza (Walkersville, MD, USA).

2.2. Cell culture

Human aortic VSMCs were obtained from Cell Applications, Inc. (San Diego, CA, USA) and used in early passage (P3-5). Cells were made quiescent by incubation in SFM for 48 h.

2.3. Migration and proliferation assays

Chemotaxis to SFM or TSP-2 or TSP-5 (1 μg/mL – 40 μg/mL) was assessed using a modified Boyden chemotaxis chamber (4 h at 37 °C). Results were recorded as cells migrated per 5 high power fields (400x). TSP-1 at a concentration of 20 μg/mL was used as a positive control, as maximal migration at this concentration was previously established [9].

For proliferation, quiescent cells were exposed to SFM or TSP-1, TSP-2 or TSP-5 (0.2 μg/mL – 40 μg/mL) for 72 h. Proliferation was assessed by MTS tetrazolium absorbance assay (Cell Titer 96 Aqueous One Solution, Promega, Madison, WI).

2.4. RNA isolation and cDNA reverse transcription

Quiescent cells were exposed to SFM, TSP-1, TSP-2 or TSP-5 (20 μg/mL) for 3, 6 or 24 h. Media was decanted and cells frozen to −80 °C. RNA was isolated with RNeasy mini kit (Qiagen, Germantown, MD). Quality of the RNA samples was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). cDNA was generated using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Grand Island, NY) using a C1000 Touch Thermocycler (Bio Rad, Hercules, CA).

2.5. Real-time quantitative reverse transcriptase-polymerase chain reaction

TaqMan® Gene Signature Human Angiogenesis Array microfluidic assay cards run on a QuantStudio 7Flex Real-time PCR system (Applied Biosystems, Grand Island, NY) were used to determine gene expression in the SFM, TSP-1, TSP-2 and TSP-5 samples at 3, 6 and 24 h.

2.6. Statistical analysis

The migration and proliferation assays were performed three times in triplicate, and analyzed by ANOVA in StatView (SAS Institute, Cary, NC). The gene expression assays were performed in duplicate as per the manufacturer's recommendation and interpreted by *t*-test using Expression Suite 7 software (Applied Biosystems, Grand Island, NY); p values < 0.05 were considered significant.

3. Results

3.1. Migration

TSP-2 induced VSMC migration at all concentrations tested ($p < 0.05$, Fig. 1). The 20 μg/mL concentration caused the greatest increase in migration by 3.11 (±0.20) fold. The 5 μg/mL and 10 μg/mL concentrations increased migration 2.26 (±0.19) – 2.60 (±0.11) fold, but were not significantly different from each other. The 1 μg/mL and 40 μg/mL concentrations induced migration 1.72 (±0.17) and 2.12 (±0.88) fold.

TSP-5 induced VSMC migration at all concentrations tested ($p < 0.05$, Fig. 1). The 20 μg/mL concentration induced the greatest increase in migration at 3.11 (±0.16) fold. The 10 μg/mL and 40 μg/mL concentrations increased migration 2.13 (±0.83) and 2.30 (±0.27) fold respectively, but were not significantly different from

Please cite this article in press as: A. Helkin, et al., Thrombospondin-1, -2 and -5 have differential effects on vascular smooth muscle cell physiology, Biochemical and Biophysical Research Communications (2015), http://dx.doi.org/10.1016/j.bbrc.2015.07.044