Stable RNA interference of host thrombospondin-1 blocks *Trypanosoma cruzi* infection

Kaneatra J. Simmons¹, Pius N. Nde¹, Yuliya Y. Kleshchenko, Maria F. Lima, Fernando Villalta*

Division of Microbial Pathogenesis and Immune Response, Department of Biomedical Sciences, School of Medicine, Meharry Medical College, 1005 Dr. D.B. Todl Jr. Bldg., Nashville, TN 37208, USA

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Abstract Interactions between *Trypanosoma cruzi* and the extracellular matrix play an important role in cellular invasion. Here we show that *T. cruzi* increases the levels of thrombospondin-1 (TSP-1) expression in host cells during early infection. Stable RNA interference of host cell TSP-1 knocks down the levels of TSP-1 transcripts and protein expression in mammalian cells causing inhibition of *T. cruzi* infection. Addition of TSP-1 to these cells restores infection. Thus, host TSP-1, regulated by the parasite, plays a crucial role in early infection. This is the first report showing that a human parasite modulates TSP-1 expression to facilitate infection.

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1. Introduction

*Trypanosoma cruzi*, the causative agent of Chagas' disease, needs to invade host cells to establish and cause infection. The parasite must navigate the extracellular matrix (ECM) in order to infect mammalian cells. Amongst the glycoproteins of the ECM, the thrombospondins constitute a unique multidomain protein that are synthesized, secreted and incorporated into the ECM by many cell types [1,2]. Thrombospondins are unique members of the ECM in that they have been described as 'matricellular' proteins because they modulate cell function but they do not play a direct role in the structure of the ECM. The five different forms of thrombospondins (TSP-1-5) are divided into two subgroups according to their oligomerization properties and molecular architecture. Subgroup A (TSP-1 and -2) are members of the thrombospondin type1(TSP1) repeat supergene family which forms homotrimers and subgroup B (TSP-3, -4 and -5) forms homopentamers [3,4]. TSP-1 contains several distinct domains, including an N-terminal heparin binding domain (HBD), a procollagen region, three type I (properdin) repeats, three type 2 (epidermal growth factor-like) repeats, type 3 repeats (calcium binding), and a C-terminal domain. All the other thrombospondins except TSP-5 have an HBD domain [5]. TSP-1 binds to specific cell-surface receptors, cytokines, proteases, and growth factors during which they help define cellular function in that environment [6,7]. TSP-1 also interacts with structural proteins such as collagens, fibronectin, and laminins [3,7]. The co-expression of one or more thrombospondins can be found in most adult tissues, and subgroup A thrombospondins are more highly expressed during cellular injury, stress and tissue remodeling. Most thrombospondin types are expressed in smooth muscle cells, fibroblasts, and endothelial cells, among others. Most tissues express at least one type of thrombospondin [1].

Previous studies have shown that *T. cruzi* molecules bind to immobilized laminin [8], and that human galectin-3 enhances this interaction [9], suggesting that the trypanosome interacts with the ECM. In addition, it was suggested that *T. cruzi* might have receptors for thrombospondin [10]. However, the role that host TSP isoforms may have in the process of microbial infections is unknown. Since nearly all cells that *T. cruzi* infects are surrounded by basement membranes, of which several TSP isoforms are important constituents, the ability of the parasite to effectively regulate and interact with TSP-1 is critically important for its passage through the membrane barrier. In this study, we test the hypothesis that *T. cruzi* binds to human cells to regulate the expression of TSP-1, which is required for *T. cruzi* infection.

In this paper we report the novel observation that early *T. cruzi* infection of human coronary artery smooth cells upregulates the expression of TSP-1 and that knocking down the expression of TSP-1 by RNA interference (RNAi) dramatically reduces *T. cruzi* infection of human cells.

2. Materials and Methods

2.1. Microorganism

The highly infective trypomastigote clone, MMC 20A derived from the Tulahuen strain of *T. cruzi* [11] was used. Pure culture trypanmastigotes were obtained from the supernatant of heart myoblast monolayers as described [11].

2.2. Cellular infection assays

Parasite binding to human cells was evaluated at 2 h by fluorescence microscopy using FITC labeled antibodies to a trypomastigote surface protein and DAPI [12]. Bound FITC-fluorescent parasites per 200 cells were microscopically determined. Parasite entry was evaluated at the
same 2 h time point. The number of internalized parasites was obtained by subtracting the number of bound FITC-fluorescent parasites from the total number of DAPI-stained kinetoplast DNA parasites per 200 host cells. Parasite multiplication within cells was evaluated at 72 h using standardized procedures [11]. In some cellular infection assays, HeLa or Vero cells were serum starved in the presence of DMEM or MEM for 24 h and then treated with or without TGF-β1 (R&D Systems, USA) at the concentration of 5 ng/ml for 24 h followed by exposure to parasites. In other assays, TSP-1 (Protein Sciences, USA) at the concentration of 8 μg/ml was added to HeLa cells transfected with construct T1 for 30 min and then used for infection assays. Infection assays using primary human coronary artery smooth muscle (HCASM) cells, HeLa and Vero cells were done in triplicate and experiments were repeated three times.

2.3. RNA extraction and quantitation of host gene expression by real-time PCR

Total RNA was extracted using TRIzol (Invitrogen, USA) and genomic DNA was removed with RQ1 (Promega, USA). First strand cDNA was synthesized using SuperScript II (Invitrogen). The cDNAs were used for real-time PCR experiments on an iCycler (Biorad, USA). The reaction mixture (25 μl) was set up as described by the manufacturer (Biorad) in triplicate. The cycling conditions for TSP-1 were as follows: 95 °C for 3 min, 40 cycles of 94 °C for 30 s and 56 °C for 45 s. We used an annealing temperature of 60 °C during which 100 continuous fluorescence readings were obtained. The quantity of human 18s RNA was used to normalize each corresponding transcription. The primers used are shown in Table 1.

### Table 1 Real-time PCR primers

<table>
<thead>
<tr>
<th>Target primer sequence (5′ → 3′)</th>
<th>GeneBank Accession Nos.</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thrombospondin-1</td>
<td>NM_003246</td>
<td>133</td>
</tr>
<tr>
<td>F: CATGCTCTGCCGTGTCCGTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T: GATGATGATGATGACAATGATGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thrombospondin-2</td>
<td>NM_003247</td>
<td>106</td>
</tr>
<tr>
<td>F: GGACCAGGAGGACACATACC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T: GTGGTCTTGCGGTTGATGTT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thrombospondin-3</td>
<td>NM_007112</td>
<td>133</td>
</tr>
<tr>
<td>F: AATGGACGGACACCGAAGCACC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T: GGACCAAGGAGGACATAATC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thrombospondin-4</td>
<td>NM_003248</td>
<td>105</td>
</tr>
<tr>
<td>F: TCAATGATGATGATGACAAATGAGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T: CCCTGCTGTCTGTCCTACCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human 18s</td>
<td>M10098</td>
<td>118</td>
</tr>
<tr>
<td>F: CGGAGACTCTCGCAGTTTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T: TGCCAGCTTCGTGCTTATCG</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

All primers were used at a final concentration of 0.2 μM. The primers were designed using the Beacon Designer Software package (Premier Biosoft, USA).

### Table 2 Oligonucleotides used for generating shRNA-targeting TSP-1 in the cells

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5′ → 3′ and antisense 3′ → 5′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1 sense</td>
<td>TCAGAGGGAGCCATGTCGACCACCACATCTCGTTG</td>
</tr>
<tr>
<td>T1 antisense</td>
<td>CCCCCGCTACGGACGCTGAGAGGACACATTGC</td>
</tr>
<tr>
<td>T1 sense scrambled</td>
<td>CCCCCGCTACGGACGCTGAGAGGACACATTGC</td>
</tr>
<tr>
<td>T1 antisense scrambled</td>
<td>CCCCCGCTACGGACGCTGAGAGGACACATTGC</td>
</tr>
<tr>
<td>T2 sense</td>
<td>TCAGAGGGAGCCATGTCGACCACCACATCTCGTTG</td>
</tr>
<tr>
<td>T2 antisense</td>
<td>CCCCCGCTACGGACGCTGAGAGGACACATTGC</td>
</tr>
<tr>
<td>T3 sense</td>
<td>TCAGAGGGAGCCATGTCGACCACCACATCTCGTTG</td>
</tr>
<tr>
<td>T3 antisense</td>
<td>CCCCCGCTACGGACGCTGAGAGGACACATTGC</td>
</tr>
</tbody>
</table>

The sense oligonucleotides were annealed with the antisense oligonucleotides and cloned into the XbaI and SalI sites (underlined) of the pCMV-800 vector. TSP-1 containing sequences represented in bold and HindIII site that separate sense from antisense shRNAs in regular text. The scrambled T1 sequences are represented in regular text.
2.6. Adhesion of transfected HeLa cells to substrate

The same concentration of transfected cells was added in triplicate to 6-well tissue culture plates. Wild-type HeLa cells were used as a control. After 4 h, the number of cells attached to wells was microscopically determined.

2.7. Presentation of results and statistical analysis

Results in this work were obtained from triplicate values and represent three independent experiments with identical protocols. Results are expressed as the means ± 1 S.D. Differences were considered to be statistically significant if \( P < 0.05 \) as determined by Student’s \( t \) test.

3. Results and discussion

Since intracellular parasites must cross the ECM and interact with cellular matrix proteins in order to attach and invade host cells, we explored the possibility that \( T. cruzi \) modulates the ECM gene expression profile in HCASM cells. We evaluated the kinetics of change in gene transcript profiles of TSP-1, TSP-2, TSP-3 and TSP-4 by real-time PCR during the early process of infection of HCASM cells by \( T. cruzi \). Fig. 1 shows that the TSP-1 transcript levels increased approximately 6-fold at 60 min, followed by an increase of 2.7-fold at 120 min during infection of HCASM cells by \( T. cruzi \). The transcript levels of TSP-2, TSP-3 and TSP-4 did not change significantly during the same period of time of \( T. cruzi \) infection of HCASM cells (Fig. 1). These results indicate that early infection of HCASM cells by \( T. cruzi \) upregulates the level of the TSP-1 transcript. \( T. cruzi \) increased the level of TSP-1 transcript in HeLa cells up to 2.1 ± 0.1-fold (\( P < 0.05 \)) at 1 h when compared to mock treated cells as evidenced by real-time PCR. Quantitative immunoblot analysis indicates that the level of TSP-1 protein in infected HeLa cells at 1 h also increased by 2 ± 0.08-fold (\( P < 0.05 \)), indicating a direct relationship between TSP-1 transcript and protein levels. The levels of TSP-1 reached normal levels at 2 h. No statistically significant changes (\( P < 0.05 \)) in TSP-2, -3, and -4 transcripts were seen in HeLa cells at 1 or 2 h.

The fact that the \( T. cruzi \) infection upregulates the expression of TSP-1 prompted us to determine the function of the TSP-1 gene in the process of \( T. cruzi \) infection of human cells by stable RNAi. We investigated whether shRNA targeted to TSP-1 could knock down its transcript. Three short nucleotide sequences derived from the TSP-1 coding sequence were cloned into IMG 800 vector (Imgenex). The sequences spanned nucleotides 1477–1496 (T1), 1758–1786 (T2), and 1546–1574 (T3). The T1 sequence was scrambled and used as an additional control. The morphology and growth rate of human cells stably transfected with the cloned constructs and selected with neomycin remained unchanged following several passages. With the aid of quantitative real-time PCR we determined the level of the TSP-1 transcripts in the transfected cells. We observed that one of the three constructs, named T1, significantly decreased the TSP-1 gene transcript by 70% as compared to the cells transfected with vector alone and scrambled T1 (Fig. 2), indicating that expression of this shRNA was effective in knocking-down the TSP-1 transcript. T2 and T3 constructs were not as effective as T1. Overexpression of TSP-1 in HeLa cells (Fig. 4C upper panel), induced by TGFβ-1, caused a significant increase in both trypanosome binding to cells at 2 h (Fig. 4C middle panel) and parasite load at 72 h (Fig. 4C, lower panel). Overexpression of TSP-1 induced by TGF-β1 was also seen in culture supernatants. These results indicate that there is a direct correlation between overexpression of TSP-1 in HeLa cells with an increase of infection of these cells. Similar results were seen when Vero cells were used instead of HeLa cells. The overexpression of TSP-1 in human cells induced by TGF-β1 is consistent with previous reports [15,16]. The enhancement of \( T. cruzi \) infection of mammalian cells caused by pretreatment of cells with TGF-β1 that we observed is also consistent with previous reports.
Furthermore, pre-exposure of HeLa cells transfected with construct T1 to exogenous recombinant TSP-1 at the concentration of 8 ng/ml for 30 min resulted in restoration of the infection of these cells by 80.4% ± 6.1 (% < 0.05).

We further investigated whether the reduction of the TSP-1 gene transcript by RNAi was reflected at the protein level by immunoblotting analysis. Our results indicate that the expression of TSP-1 in human cells stably transfected with the T1 construct was significantly reduced as compared to wild type cells, cells transfected with vector alone or scrambled T1 (Fig. 3A). Determination of the relative absorbance of TSP-1 and β-actin bands showed that RNAi of TSP-1 significantly decreased the protein level by 89% (Fig. 3B). The expression of other ECM proteins (fibronectin and laminin) and galectin-3 in transfected HeLa cells remained unchanged as evidenced by immunoblots (Fig. 3C). We also observed that transfected cells showed no differences in adhesion to substrate compared to wild type HeLa cells (Fig. 3D). Furthermore, immunofluorescence studies of transfected and wild type cells probed with anti-fibronectin, anti-laminin and anti-galectin-3 antibodies showed no significant differences in the expression of these proteins with respect to wild type, cells transfected with scrambled T1 or vector alone.

To determine the function of TSP-1 in the process of T. cruzi infection, we exposed human cells presenting the new phenotype to invasive trypomastigotes. We observed that RNAi of TSP-1 dramatically reduced the number of trypomastigotes that attached to human cells stably transfected with the T1 construct compared to cells transfected with vector alone and scrambled T1. Human cells expressing the effective shRNA had less parasites bound per cell as compared to mock-transfected cells at 2 h of T. cruzi infection (Fig. 4A). We also observed that cells transfected with the T1 construct presented a low parasite load at 72 h as compared to cells transfected with vector alone or scrambled T1. Cells transfected with construct T1 presented a reduced infection rate at 72 h up to 58% ± 7.5 (% < 0.05) with respect to cells transfected with vector alone or scrambled T1.

The fact that less trypanosomes bind to cells stably transfected with the T1 construct (Fig. 4A) at 2 h consequently caused less parasites to enter and multiply within these cells at 72 h with respect to cells transfected with vector alone or vector containing scrambled T1 (Fig. 4B). Analysis of parasite multiplication at 72 h indicated that cells transfected with vector alone and vector containing scrambled T1 had a higher number of parasites per cell, whereas cells transfected with the T1 construct had significantly lower number of parasites per cell. These results indicate that RNAi of TSP-1 substantially reduced the transcript and its encoded protein, rendering the hu-
man cells to be substantially less susceptible to *T. cruzi* infection. Our findings, showing that RNAi of TSP-1 causes a dramatic reduction of *T. cruzi* infection of human cells, indicates that TSP-1 is required for the process of *T. cruzi* infection. The residual *T. cruzi* infection seen indicates that the parasite may use other TSP-1 independent mechanisms to infect cells.

*T. cruzi* infection causes extensive fibrosis and severe cardiomyopathy in the heart, which is in part vasculopathy, leading to cardiac arrest that is frequently followed by death [19]. *T. cruzi* must navigate through the basal lamina, which contains TSP-1, and surrounds individual muscle cells such as HCASM cells before infecting these cells. The fact that *T. cruzi* trypomastigotes increased TSP-1 transcripts in HCASM cells suggest that TSP-1 may contribute in part to the pathology caused by *T. cruzi*.

Previous studies in our laboratory have shown that *T. cruzi* trypomastigote gp83, a molecule that the parasite uses to attach to host cells, upregulates laminin γ1 [20], whereas others have shown that *T. cruzi* uncharacterized antigens upregulate laminin, fibronectin, and type I collagen [21].

Our observations that *T. cruzi* remodels the ECM by upregulating the expression of TSP-1 indicate that the parasite exploits TSP-1 to navigate through the ECM to facilitate infection. Here we show that TSP-1 is required for the infection process of *T. cruzi* as evidenced by RNAi of that specific isoform.

In summary, we report the novel findings that *T. cruzi* upregulates the expression of host TSP-1 which facilitates the infection of human cells. Thus, *T. cruzi* modifies TSP-1 expression in the ECM to contribute to the pathogenesis of *T. cruzi* infection.

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References

