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13. ABSTRACT (Maximum 200 Words)

ADAMTS1 is a secreted metalloprotease with a disintegrin-like motif and three type-1 thrombospondin domains. ADAMTS1 has been shown to inhibit both endothelial cell proliferation in vitro, as well as angiogenesis in vivo. However, inactivation of the catalytic domain of this protease suppressed its inhibitory activity. Hence, it was important to identify substrates for ADAMTS-1 to gain insight into its mechanism of action. We have identified Thrombospondin-1 (TSP-1) as a novel substrate of ADAMTS-1. This study demonstrated that incubation of TSP-1 with ADAMTS-1 results in a single cleavage adjacent to the procollagen homology domain. Proteolysis by ADAMTS-1 releases a C-terminal fragment from a trimerized N-terminal fragment. Analysis of TSP-1 within T47D breast carcinoma tumors reveals that ADAMTS-1 is able to cleave TSP-1 in vivo. Studies using recombinant TSP-1 fragments demonstrated that such fragments are active and possess anti-migratory, anti-proliferative activity (Elzie and Murphy-Ullrich, 2004; Iruela-Arispe et al., 1999; Murphy-Ullrich et al., 1993). We predict that cleavage of TSP-1 by ADAMTS-1 results in the release of bioactive TSP fragments with the potential to suppress angiogenesis. Overall these findings predict an additional mechanism for the inhibitory effects of ADAMTS-1 in tumor angiogenesis.

14. SUBJECT TERMS
Angiogenesis, tumor biology, endothelial cells, angiogenesis inhibitors

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Introduction

Growth tumors and metastasis require neovascularization. The dependency of tumors on blood vessels has been clearly demonstrated by recent clinical trials in which suppression of tumor growth has been accomplished by inhibitors of angiogenesis. Consequently further investigation on novel inhibitors and a full understanding of their mechanism of action can bring new avenues of therapy for the treatment of tumors. Using the anti-angiogenic domain of thrombospondin-1 (TSP1), several years ago our laboratory cloned two novel human proteins: METH1/ADAMTS1 and METH2/ADAMTS8 (Vazquez et al., 1999). In addition to several thrombospondin anti-angiogenic domains in the carboxy-terminus (three for ADAMTS1 and two for ADAMTS8), these proteins also contain an ADAM cassette, which includes a metalloprotease domain and a disintegrin motif. The proteins are in fact, active metalloproteases (Rodriguez-Manzaneque et al. 2002; Sandy et al. 2001) that are secreted as zymogens and require removal of the pro-domain to become active (Rodriguez-Manzaneque et al., 2000). Our laboratory has demonstrated that these proteins have anti-angiogenic properties in several in vivo and in vitro bioassays affecting growth factor signaling (Luque et al. 2003, Vázquez et al. 1999).

During this last year we have also found that TSP1 is a substrate for ADAMTS1. Processing of TSP1 by ADAMTS1 results in the release of anti-angiogenic peptides that are likely to contribute to the tumor growth suppression mediated by ADAMTS1. Finally we have developed adenoviral vectors for the delivery of ADAMTS1 protein and showed that these are extremely effective in inhibition of tumor growth. This work has completed the objectives presented three years ago in this fellowship application.

Body

1)TSP-1 is cleaved by ADAMTS-1. Previous studies on the effects of ADAMTS-1 in tumor growth and angiogenesis revealed that the catalytic activity of this protein was essential for its function (Iruela-Arispe et al., 2003 and previous report). We have found thrombospondin-1 (TSP-1) to be a novel substrate of ADAMTS-1. Analysis of the fragments by electrophoretic mobility and Western blot techniques revealed two smaller polypeptides of 125 kDa and 36 kDa in size (figure 1). This cleavage by ADAMTS-1 appeared specific to TSP-1 since at the same molar ratio, laminin was not cleaved by ADAMTS-1 (data not shown). To confirm the specificity of the cleavage of TSP-1 by ADAMTS-1, a catalytically inactive ADAMTS-1 (E385A) and the carboxy terminal tail of ADAMTS-1 were incubated with TSP-1 in parallel. TSP-1 was only cleaved by the active ADAMTS-1 protease and not by inactive ADAMTS-1 (E385A) nor by the carboxy terminal fragment (figure 1).

In addition, adenoviral constructs expressing active ADAMTS-1, inactive ADAMTS-1 or GFP (generated in collaboration with Dr. Lilly Wu, UCLA) were used to infect 293T cells and evaluate cleavage of TSP-1. Conditioned medium (CM) was collected 4 hrs post-infection and it was subsequently incubated with either purified human TSP-1 or mTSP-1 from mouse LE II cells at 37° C for 2 hours. Under these conditions, mTSP-1 was also cleaved by ADAMTS-1 releasing a fragment 36 kDa in size indicating that the cleavage site was likely to be the same between the two proteins (figure 2).

2) ADAMTS-1 cleaves TSP-1 in an unique site within the procollagen domain. Analysis of the TSP-1 fragments resulting from ADAMTS-1 proteolysis with MALDI-TOF mass spectrometry determined that the 36 kDa fragment is the amino-terminal fragment and the 125 kDa fragment is the carboxy terminal fragment. To determine the exact site of cleavage, the 125 kDa fragment was subjected to N-terminal Edman degradation sequencing. Edman sequencing revealed that one of the resulting peptides had LRRPPL as its N-terminal sequence indicating that cleavage occurred between residues: glutamic acid 311 and leucine 312. This site is consistent

with the classification of glutamyl endopeptidase attributed to ADAMTS-1 and based on cleavage of other substrates such as aggrecan and versican (Rodriguez-Manzaneque et al., 2002; Westling et al., 2002).

3) Cleavage of TSP-1 is not shared by ADAMTS-4. ADAMTS-1 and ADAMTS-4 display high sequence homology and more importantly, both proteases share substrates (aggrecan and versican). Thus, we sought to determine whether TSP-1 was also cleaved by ADAMTS-4. Nonetheless, at the same molar concentrations, TSP-1 was not cleaved by ADAMTS-4 (figure 3A). To ensure that both proteases were catalytically active, aggrecan was digested with both ADAMTS-1 and ADAMTS-4 (figure 3B). As expected, both were able to cleave aggrecan, although ADAMTS-4 was a more effective enzyme for aggrecan than ADAMTS-1.

4) ADAMTS-1 cleaves TSP-1 in vivo. We have demonstrated that ADAMTS-1 can inhibit tumor angiogenesis in breast carcinoma xenograft tumor assays and that the proteolytic activity is essential to this function (Iruela-Arispe et al., 2003). To determine whether ADAMTS-1 cleaves TSP-1 in the xenograft assays, tumor lysates were analyzed by Western blot using two different antibodies to TSP-1. The polyclonal antibody GPC revealed two bands of 50 kDa and 36 kDa that were not as abundant in tumors expressing truncated (TRNC) or inactive (E385A) ADAMTS-1 protein (figure 4.A). The 36 kDa band may be the N-terminal fragment. The same membrane was stripped and reprobed using the monoclonal antibody Ab-4. This antibody revealed two bands of 50 kDa and 28 kDa in molecular weight that were present in truncated and inactive ADAMTS-1 expressing tumor lysates, but not present in tumor lysates expressing active ADAMTS-1 (figure 4.B). In all tumors, full length TSP-1 was not visible, perhaps due to cleavage by various proteases expressed the tumors. Western analysis with enolase ensured that lysates were loaded equally (figure 4.C). Together, these differential banding patterns, suggest that TSP-1 is proteolyzed by active ADAMTS-1 within the tumors.

5) Adenoviral delivery of ADAMTS1 protein suppresses tumor growth. AdCMV-METH1 and AdCMV-TSP1, were generated in collaboration with Dr. Lily Wu's laboratory at UCLA. Virus

were titered by plaque assays on 293 monolayer cells (infectious units = plaque-forming units). Tumor cells (CWR22Rv1) were infected at 10 infectious units/cell, harvested and lysed using RIPA lysis buffer (10 mM Tris, 150 mM NaCl, 0.1% SDS, 1% DOC, 1 mM EDTA and 1% NP40) and 10ug of protein per lane were analyzed. We selected two colonies for further evaluation: a high producer (METH-1 H) and a low producer (METH-1 L) for comparison. Subsequently, tumor cells were injected subcutaneously in the flank of nude mice and tumor growth was monitored by in vivo luciferase expression (as the tumor cells constitutively expressed CMV-luciferase). Thus, luciferase units correspond to tumor cell viability. The CCD images were obtained every four days using a cooled IVIS CCD camera (Xenogen, Alameda, California) and images were quantified. Within the linear range of signal intensity (< saturation limit of 65,000 RLU), we determined that the maximum RLU/min within a region of interest (ROI) to be the most consistent for comparative analysis and that the results correlated closely with luminometry. Figure 5 shows images of a 5 animal co-hort injected with tumor cells expressing empty vector, TSP1, METH1 (High) and METH1 (Low) at day 47 (terminal end-point). Kinetics of tumor growth is shown in Figure 6. As it can be observed, METH1 (ADAMTS1) is extremely effective in suppressing tumor growth. These results support experiments performed in vivo and confirm the potential exploration of METH1/ADAMTS1 for the suppression of angiogenesis in tumors.

Figure 1. **Figure 2.**

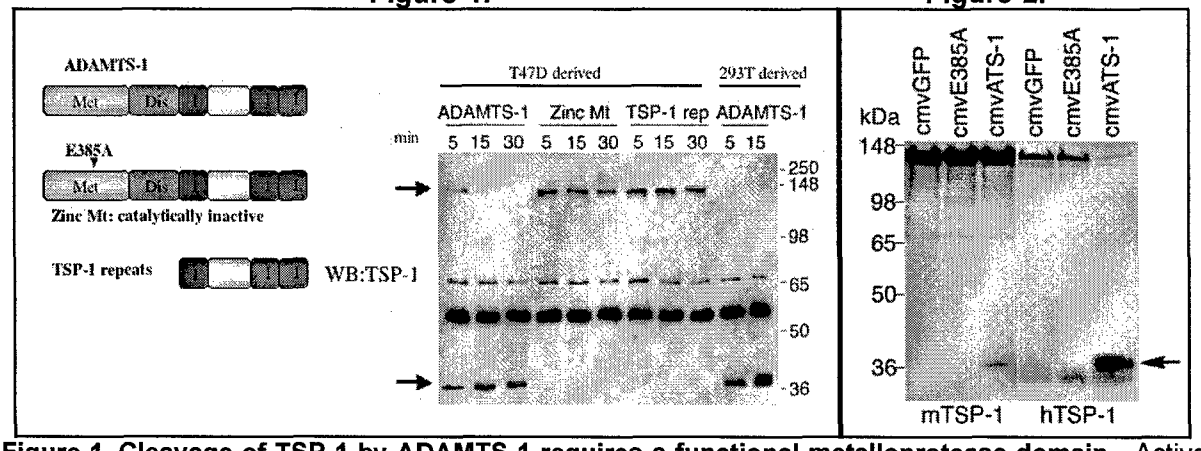

Figure 1. Cleavage of TSP-1 by ADAMTS-1 requires a functional metalloprotease domain. Active ADAMTS-1 protease derived from both T47D and 293T cell lines cleaves TSP-1 within 5 minutes at a

1:1 substrate:enzyme molar ratio. In contrast, neither the catalytically inactive mutant nor the TSP-1 repeats, alone were able to cleave TSP-1.

Figure 2. Murine TSP-1 is also a substrate for ADAMTS-1. Murine TSP-1 and purified human TSP-1 was incubated with conditioned medium of 293T cells infected with adenovirus containing GFP, catalytically inactive ADAMTS-1 (E385A), or wild type ADAMTS-1. In both cases, the fragments recognized by anti-TSP-1 antibodies appear to be the same molecular weight suggesting that the murine TSP-1 cleavage site is similar to the human TSP-1 cleavage site.

Figure 3.

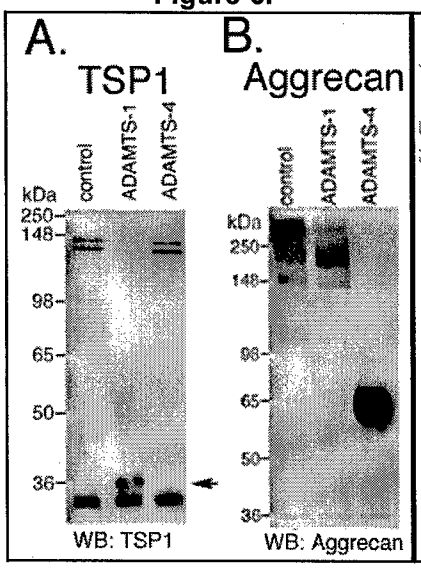

Figure4.

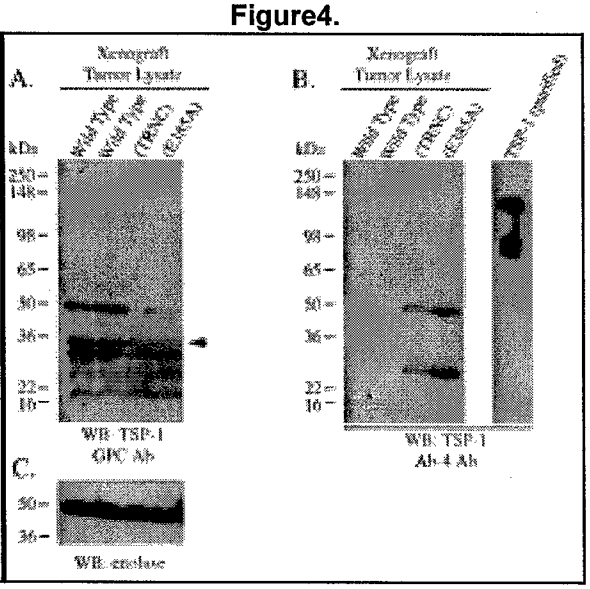

Figure 3. TSP-1 was efficiently cleaved by ADAMTS-1, but not by ADAMTS-4. ADAMTS-1 and ADAMTS-4 were incubated at the same molar concentrations with TSP-1 to determine if this substrate is also shared. Interestingly, ADAMTS-4 does not cleave TSP-1 (fig 3A). Arrow is showing TSP1 cleavage product by ADAMTS1. Catalytic activity of ADAMTS-1 and ADAMTS-4 was present as demonstrated by aggrecan cleavage (fig 3B).

Figure 4. ADAMTS-1 cleaves TSP-1 in vivo. A. Western analysis of TSP-1 (GPC). This antibody recognizes the amino terminus of TSP-1. Black arrow points to the 36 kDa fragment. **B.** Tumor lysates were evaluated with a mouse monoclonal TSP-1 antibody (Ab-4) that recognizes the procollagen homology domain. The fragments recognized by this antibody are not present in wild type ADAMTS-1 tumors. Truncated ADAMTS-1 seems to contain intermediate catalytic activity. **C.** Enolase levels show even loading of proteins.

Figure 5

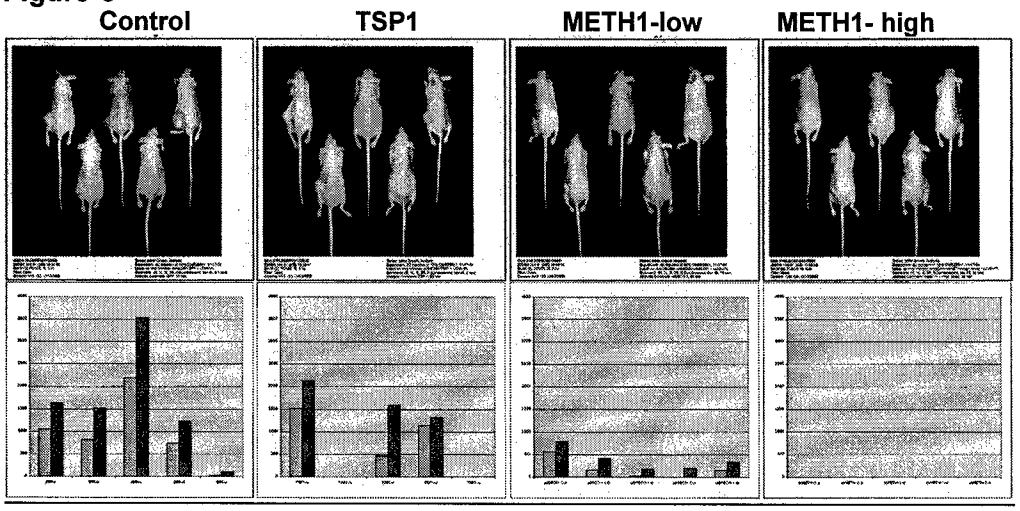

Figure 5 - Adenoviral vector-mediated gene delivery of anti-angiogenic therapy. CCD images of mice injected with cells expressing either AdCMV-control, TSP1, or METH1(L and H). The images represent the results from 5 animals of each cohort at 47 d post injection. The maximal signal intensity (maximum RLU/min) is shown below each image. The acquisition times were reduced to offset saturated liver signal intensities in AdCMV-luc cohort.

Figure 6. A. **B.**

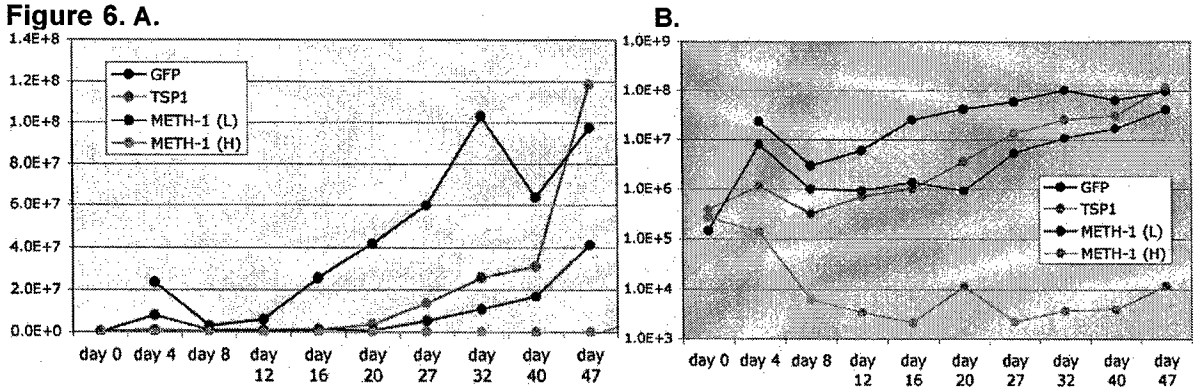

Figure 6 - Kinetics of tumor growth as determined by luciferase readings of tumor viability. The relative light intensity (RLU/min) emitted from the animal was quantified by image analysis software and depicted in the graphs (A. Linear; B. Logarithmic). The acquisition times were reduced to offset saturated liver signal intensities in AdCMV-luc cohort. Evaluation was determined every 4 days.

Key Research Accomplishments

Annual Summary 2004-2005 for Award Number DAMD17-02-1-0329
Proposal Title: Angiogenesis inhibitors in Breast Cancer
Principal Investigator: Alfonso Luque, Ph.D., UCLA

In this Annual Summary I summarize the research accomplished for the period of March 25, 2004 - March 24, 2005 under the Grant number DAMD17-02-1-0329. The results complete the 1st objective proposed: "to determine the mechanism of action of METH1 that affects endothelial cell proliferation" and the 2nd objective: "to ascertain the effect of METH-1 over-expression in the suppression of mammary tumors in vivo". In summary, we provide evidence to support the function of ADAMTS-1 as a potent inhibitor of tumor growth by an anti-angiogenic mechanism. This involves the processing of the extracellular matrix protein TSP-1 releasing bioactive TSP fragments with the potential to suppress angiogenesis. In addition, adenoviral vectors expressing full length and the catalytic inactive forms of ADAMTS1 were generated and tested for the production and function of the protease as well as for their effect in tumor growth. Currently, we are in the process of writing two manuscripts for publication with acknowledging of this grant.

Reportables Outcomes

a) Peer review manuscripts.

* Luque A, Carpizo DR, Iruela-Arispe ML. ADAMTS1/METH1 inhibits endothelial cell proliferation by direct binding and sequestration of VEGF165. J Biol Chem. 2003 Jun 27;278(26):23656-65.

* Iruela-Arispe ML, Carpizo D, Luque A. ADAMTS1: a matrix metalloprotease with angioinhibitory properties. Ann N Y Acad Sci. 2003 May;995:183-90

* Iruela-Arispe ML, Luque A, Lee N. Thrombospondin modules and angiogenesis. Int J Biochem Cell Biol. 2004 Jun;36(6):1070-8.

* Lee N, Rodriguez-Manzaneque, J.C., Twal W.O, Luque, A., Argraves, W.S. and Iruela-Arispe, M.L. Fibulin-1 acts as a cofactor for the matrix metalloprotease ADAMTS-1. Submitted.

b) Poster and oral presentations.

* Lee, N., Rodríguez-Manzaneque, J.C., Twal, W.O., Luque, A. Agraves, W.S. and Iruela-Arispe, M.L. "The proteolytic actvity of ADAMTS1 is modulated by fibulin-1". The American Society for Matrix Biology. Houston, Texas-2002.

* Luque, A. Carpizo, D. and Iruela-Arispe, M.L. "ADAMTS1 inhibits endothelial cells proliferation by direct binding and sequestration of $VEGF_{165}$".Gordon Research Conference: Vascular Biology. Ventura, California-2003.

*Oral presentations disclosing some of the results developed during this last year:

-Fourth Interdisciplinary Euroconference on Angiogenesis, Helsinki, Finland, May 2004.
-Vasculata, Course on Vascular Biology, Seattle, July 2004.
-Mayo Clinic Symposium on Angiogenesis, October, 2004

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