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Award Number: DAMD17-02-1-0329

TITLE: Angiogenesis Inhibitors in Breast Cancer

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REPORT DATE: April 2004

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	April 2004	Annual Summary (25 Mar 2003 - 24 Mar 2004)

4. TITLE AND SUBTITLE

Angiogenesis Inhibitors in Breast Cancer

5. FUNDING NUMBERS

DAMD17-02-1-0329

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

ADAMTS1/METH1 is a secreted protease that belongs to the metallospondin/ADAMTS sub-family of the zinc-metalloprotease superfamily. This family is characterized by proteins that contain a modular structure that includes **A**, **D**isintegrin-like, **M**etalloprotease, and type-1 **T**hrombo**S**pondin domains. ADAMTS1 has been shown to inhibit both endothelial cell proliferation in vitro, as well as angiogenesis in vivo. We have investigated the role of ADAMTS1 and its proteolytic activity, through the creation of a catalytically inactive mutant ($ADAMTS1^{E385A}$), in tumor growth by overexpressing the coding region in a human breast carcinoma cell line (T47D) and generating xenograft tumors in nude mice. T47D $ADAMTS1^{E385A}$ tumors displayed no significant difference in growth kinetics when compared to CON tumors; whereas wild-type ADAMTS1 tumors exhibited two-fold growth inhibition at 40 days post-implantation. We have also studied the activity of ADAMTS1 on endothelial cells in vitro and have determined that ADAMTS1 releases proteins from the cell surface. We also provide evidence to support that the catalytic activity of ADAMTS1 either directly or indirectly affects growth factor signaling impairing VEGF:VEGFR2 interactions both in vitro and in vivo. Thus ADAMTS1 can be distinguished as a secreted metalloprotease that inhibits tumor growth and angiogenesis through its activity as a protease.

14. SUBJECT TERMS

Angiogenesis, tumor biology, endothelial cells, angiogenesis inhibitors

15. NUMBER OF PAGES

10

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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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).

Results

1) The anti-tumor and anti-angiogenic effects of ADAMTS-1 are dependent on its catalytic activity. To investigate the relative contribution of the metalloprotease domain to the anti-angiogenic properties of ADAMTS1 we have generated a mutant construct (ADAMTS1^{E385A}) that contains a single amino acid substitution in the zinc-binding pocket of the metalloprotease domain rendering the protein catalytically inactive. Recombinant protein from this construct has been verified as catalytically inactive via an in vitro activity assay based on aggrecan cleavage (Rodriguez-Manzaneque et al. 2002). The effect of ADAMTS1^{E385A} expression on tumor growth was evaluated with respect to both CON and wild-type ADAMTS1 by generation of xenograft tumors in nude mice using a human breast carcinoma cell line (T47D) overexpressing ADAMTS1, ADAMTS1^{E385A} or vector alone. T47D ADAMTS1^{E385A} tumors displayed no significant difference in growth kinetics when compared to CON tumors; whereas wild-type ADAMTS1 tumors exhibited two-fold growth inhibition at 40 days post-implantation (figure 1).

2) ADAMTS1 cleaves Syndecan 4. Given the importance of the catalytic activity of ADAMTS-1 to its anti-angiogenic properties, we investigated the hypothesis that ADAMTS1 targets proteins at the surface of the endothelium that either directly or indirectly function to inhibit angiogenesis. To test this hypothesis we submitted to mass spectrometry the biotinylated fragments released from the surface of endothelial cells incubated with ADAMTS1 (figure 2). The analysis of the sequences revealed Syndecan 4 like a possible candidate. We corroborated this result in vitro with co-transfection experiments (figure 3), and immunocitochemistry (data not shown).

3) Syndecan 4 binds to VEGF. VEGF binds to heparan sulfate on the cell surface and extracellular matrix (Cross et al. 2003). Here we described, by the first time, interaction of VEGF to Syndecan 4 in a crosslinking approach (figure 4).

4) Catalytic activity of ADAMTS1 decreases VEGF binding to the cell surface and Syndecan 4 expression on the cell surface. ADAMTS1 treatment, but not ADAMTS-^{E385A}, significantly decreased the level of bound ^{125}I-VEGF to the endothelial cells (data not shown). In accordance with that result, when we subjected tumor cryosections to immunostaining using an antibody that recognizes VEGF only when it is complexed with the VEGFR2 receptor, we found there to be a significantly diminished level of staining on the surface of the tumor vasculature than was seen in controls (data not shown). On the other way, immunohistochemistry using an antibody to the Syndecan-4 ectodomain reveals a diminished level of staining on both the tumor cell and endothelium in ADAMTS1 tumor sections compared to the catalytic inactive ADAMTS^{E385A} (figure 5).

Figure 1)

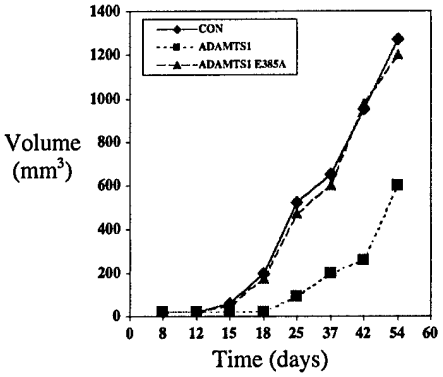

Figure 2)

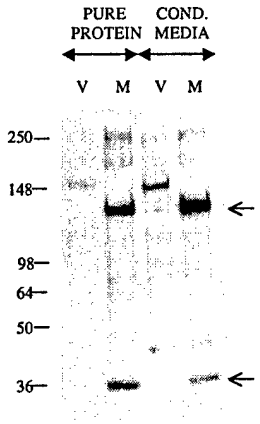

Figure 3)

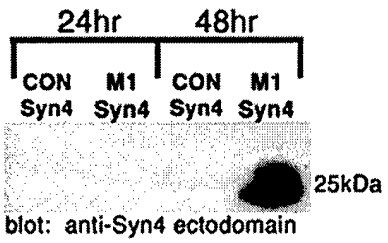

Figure 1) The anti-tumor and anti-angiogenic effects of ADAMTS-1 are dependent on the catalytic activity. Tumor growth kinetics of T47D CON, ADAMTS1 and ADAMTS1 ^{E385A} tumors demonstrating the loss of growth suppression in the ADAMTS1 ^{E385A} tumors that is evident in the wild-type ADAMTS-1 tumors (n=3). **Figure 2) ADAMTS1 cleaves proteins from BAEC surface.** BAEC were biotinylated according to standards procedures and treated with vehicle (V), conditioned media containing ADAMTS1 (A) or 3μg/ml of purified protein (M) for 2h at 37°C. Cells were lysed and biotinylated proteins from the conditioned media and from the cell lysates were purified with avidin-agarose-beads. The samples were subjected to SDS PAGE and immunobloted. Shed molecules were detected using avidin-horseradishperoxidase. Released proteins are marked with arrows. **Figure 3) ADAMTS1 is a Syndecan-4 sheddase.** T47D control (CON) and ADAMTS1 (M1) cells tranfected with wild type Rat Syndecan-4 were plated in the absence of serum. Proteins from the serum free media were collected at 24 and 48-hour time

points, subjected to glycosaminoglycan digestion followed by Western analysis using an antibody to the ectodomain of Syndecan-4.

Figure 4)

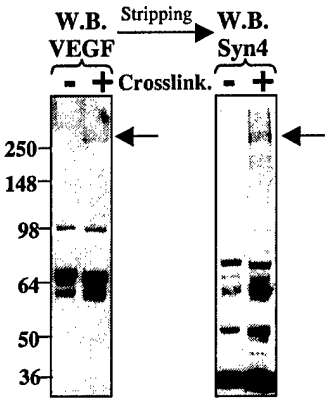

Figure 5)

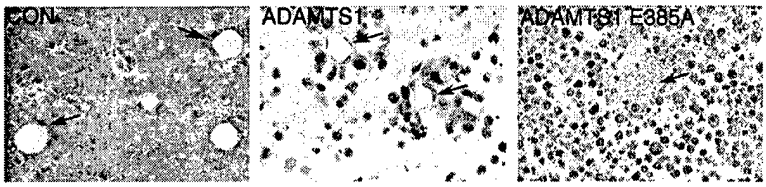

Figure 4) $VEGF_{165}$ interacts with Syndecan4. After incubation of T47D cells with $VEGF_{165}$, the cells were washed and the interacting proteins on the cell surface were crosslinked using disuccinimydil suberate and the complexes formed were analyzed by Western-blot techniques with anti-VEGF antibodies. Levels of syndecan4 were evaluated reprobing the same membrane with specific antibodies. Non-crosslinked samples were used as control. *Arrow* indicates the specie recognized by both antibodies. **Figure 5) Syndecan4 expression on ADAMTS1 and catalytic inactive ADAMTS1 xenografts tumors.** Tumor sections stained with an antibody to the Syndecan-4 ectodomain reveals a diminished level of staining on both the tumor cell and endothelium in ADAMTS-1 tumor sections compared to the ADAMTS1 ^{E385A} or control (CON).

Key Research Accomplishments

Annual Summary 2003-2004 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 present the research accomplished for the period of March 25, 2003 - March 24, 2004 under the Grant number DAMD17-02-1-0329. These results partially address 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 this study, we provide evidence to support that ADAMTS-1 can function as a potent inhibitor of tumor growth by an anti-angiogenic mechanism that involves the release of endothelial cell surface molecules that indirectly inhibit VEGF:VEGFR2 interactions. These findings have been described in two reviews (Iruela-Arispe et al. 2004 and 2003) with acknowledge of this grant. In this regard the described results on this research is currently under preparation and will shortly be submitted for publication.

Reportables Outcomes

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

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