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Role of the Thrombospondin - CD36 - Histidine Rich Glycoprotein Pathway in Tumor Growth and Angiogenesis

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ROLE OF THE THROMBOSPONDIN - CD36 – HISTIDINE RICH GLYCOPROTEIN PATHWAY IN TUMOR GROWTH AND ANGIOGENESIS

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DEDICATION

To Laura Vanlandingham Hale

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ABSTRACT

Cancer is typically thought of as an uncommon disease, in which solid tumors require a blood supply in order to grow and metastasize. Interestingly, upon autopsy a large portion of elderly individuals display numerous non-vascularized lesions throughout their bodies. Thus, the angiogenic switch in the development of cancer presents an important therapeutic target. Previous work by our laboratory has established an interaction between CD36, Histidine Rich Glycoprotein (HRGP) and Thrombospondin 1 (TSP-1) in the modulation of angiogenesis. Briefly, endothelial cell receptor CD36 interaction with soluble or cell bound TSP-1 leads to the induction of an apoptotic signaling cascade in vascular endothelial cells resulting in decreased proliferation, migration and tube formation, thereby inhibiting angiogenesis. Presence of soluble HRGP leads to inhibition of the anti-angiogenic potential of the CD36-TSP-1 pathway through a decoy receptor function whereby TSP-1is bound and sequestered. Previous studies have focused on this pathway with regards to wound healing. However, pathologically relevant modulation of angiogenesis is also observed in tumors. In the current work we evaluate the role of the CD36-TSP-HRGP pathway in tumor growth and angiogenesis. Further, we examine a possible processing mechanism by which TSP function may be modulated by a matrix metalloprotease, ADAMTS1.

Chapters two through five will outline the role of the TSP-CD36 axis in tumor biology, namely angiogenesis and growth. We will also address modulation of this

pathway via HRGP. Further we will describe a matrix metalloprotease mechanism by which TSP function may be regulated.

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CHAPTER I

INTRODUCTION

Cancer Overview

Cancer as defined by the American Cancer Society is a group of diseases characterized by the uncontrolled growth of abnormal cells, which if not restricted may result in death[1]. Extrinsic/environmental factors including UV radiation, tobacco use and chemical exposure and intrinsic factors such as immune function, genetic background and hormone levels may cause or hasten the induction of cancerous lesions[2,3,4,5,6,7]. This group of diseases is now recognized as one in which multiple genetic "hits" or mutations are required for initiation.

These mutations result in the induction of oncogenes or inhibition of tumor suppressor genes. Proto-oncogenes are normal genes that may become oncogenes upon mutation or over-expression. These genes encode proteins that regulate cell division and growth and include RAS, WNT, MYC and ERK[8,9]. Tumor suppressor genes are native genes that regulate cell division, repair deoxyribonucleic acid (DNA) and control apoptosis (controlled cell death). Upon mutation, these genes are inhibited resulting in decreased action and uncontrolled cell growth. Common tumor suppressor genes include p53, BRCA1, APC and RB1[10]. It is through a combination of oncogene activation an

tumor suppressor inhibition that cells may acquire the mutations necessary to take on a malignant phenotype and initiate cancer.

These mutations result in a set of functional capabilities better known as the hallmarks of cancer, as proposed by Hanahan and Weinberg[11]. This includes evasion of apoptosis, self sufficiency in growth signals, insensitivity to anti-growth signals, limitless replicative potential, sustained angiogenesis and tissue invasion and metastasis. These functional capabilities allow cells to break free from the constraints of their microenvironment resulting in uninhibited growth and the formation of cancerous lesions.

Disease Statistics and Treatments

The American Cancer Society estimates that males in the United States have a 1 in 2 chance of developing cancer over their lifetime, with females experiencing a lower rate of 1 in 3. Cancer is the second leading cause of death in the United States, behind heart disease. Further, for 2011 it is anticipated that 571,950 individuals will die as a result of cancer with an additional 1,596,670 new cases being diagnosed[1].

Currently, there are numerous therapeutic strategies for cancer as a whole. Most often these strategies are used in concert rather than individually. These include resection of the cancerous lesion (at this time the only effective strategy for melanomas)[12], radiation therapy (more specifically ionizing radiation which dislodges electrons leading to cell death)[13], chemotherapy (in which injected or ingested antimetabolites or inhibitors lead to cell death in rapidly dividing cells)[14], immunotherapy (where the hosts immune system is activated by manufactured antibodies or vaccines, gardasil for example to prevent cervical cancers)[15], photodynamic therapy (most often used with skin cancers

whereby light causes a photosensitizing agent to interact with oxygen and produce free radicals toxic to the cell)[16], thermal therapies (in which heat or cold are used to destroy small areas of cells)[17,18] and lastly anti-angiogenic therapies **(**which take two forms, destruction of existing vessels or prevention of the formation of new ones).

Unfortunately, the majority of these therapies are relatively nonspecific, targeting both healthy host and tumor tissue. As such, there is an urgent need for treatments based on molecular pathways or specific antigens, some of which are more prevalent in individual cancers; breast cancer 1 (BRACA1) in breast cancer and melanoma and adenomatous polyposis coli (APC) in colon cancer for example. Significant strides have been made utilizing the genomic approach of cancer treatment; we will focus on several advancements in targeted cancer treatment with regards to angiogenic modulation.

In 1999, the National Cancer Institute marked the development of angiogenic inhibiting cancer therapies as an urgent priority. Following, in 2004, Bevacizumab, a humanized monoclonal antibody against VEGF, better known as Avastin became the first anti-angiogenic compound approved by the FDA for the treatment of cancer[19]. As of March 2011, four anti-angiogenic drugs have been approved by the FDA, all of which target the vascular endothelial growth factor (VEGF) pathway[20].

Currently, several angiogenesis inhibitors are in phase III clinical trials for varied human cancers including melanoma, breast and gastric cancer[21]. Trial NCT00111007 is currently examining the use of Sorafenib in combination with chemotherapeutics Paclitaxel and Carboplatin in the treatment of Stage III and IV melanoma. Sorafenib is a kinase inhibitor shown to inhibit angiogenesis through the induction of endothelial cell apoptosis[22]. Trial NCT01303679 is currently evaluating the use of Avastin in

combination with chemotherapeutics Taxane and Exemestane in the treatment of metastatic breast cancer. Avastin is currently under study in approximately one dozen additional phase III clinical trials. Trial NCT01170663 is currently evaluating the use of Ramucirumab in combination with chemotherapeutic paclitaxe in the treatment of gastric carcinoma. Ramucirumab is a human monoclonal VEGFR2 activating antibody. It is one of two making use of Ramucirumab, with a third examining Avastin.

These trials use a combinatorial approach, targeting the vasculature along with the rapidly dividing tumor cell population. Despite the promise these trials present, we should not "put all our eggs in one basket". As such, additional avenues of angiogenic modulation should be examined.

Angiogenesis and Cancer

Angiogenesis is the physiologic process by which new vessels sprout from the existing vasculature. In 1907 the association of the vasculature and solid tumors was first described[23]. However, the field of angiogenic research did not begin until 1971 with the publication of work by Judah Folkman hypothesizing the growth of neoplastic lesions was dependent on angiogenesis[24], which has since been validated in numerous tumor types including those originating in the brain, breast, prostate, skin and lung[25,26,27,28,29].

In the normal adult setting, the vasculature is maintained in a quiescent state through a balance of angiogenic inhibitors, such as Thrombospondin 1 (TSP), and inducers, such as VEGF. This balance between pro- and anti-angiogenic stimuli is important in homeostasis, in particular in such conditions as pregnancy and wound healing. Loss of homeostatic balance resulting in excessive or insufficient angiogenesis has been implicated in numerous diseased states such as ulcerative colitis, diabetic

retinopathy, obesity, psoriasis, rheumatoid arthritis, stroke, coronary artery disease and cancer[30,31,32,33,34,35,36].

Interestingly, most apparently healthy individuals display numerous small nonvascularized lesions throughout their bodies. However, only approximately 1 in 600 of these small, quiescent tumors will acquire an angiogenic phenotype resulting in a clinically detectable cancer[37]. Supporting this observation, it is well established that solid tumors will grow to 1-2 mm by simple diffusion but require a blood supply in order to expand further and metastasize. To this end tumors express pro-angiogenic substances such as basic fibroblast growth factor (bFGF) and VEGF which recruit blood vessels to the lesion through the induction of endothelial cell proliferation migration and tube formation (Figure 1).

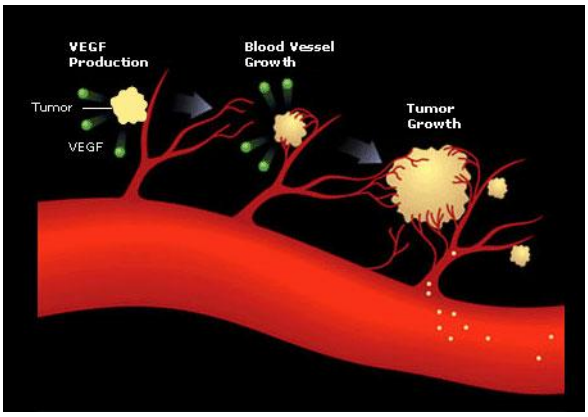

Figure 1. **Tumor vessel recruitment**. Tumors will grow to a limited volume through diffusion, 1-2 mm. In order to expand further they secrete angiogenic compounds, such as VEGF, allowing for the recruitment of blood vessels and subsequent growth and metastasis (Genentech).

Cardiovascular Structure

The cardiovascular system supplies our tissues with oxygen and nutrients as well as collecting and expelling carbon dioxide (CO_2). Most cells of the body are located within 200 μm of a blood vessel, the diffusible limit for oxygen[38]. The heart is the engine that drives blood flow throughout the body. Deoxygenated blood enters the right atrium

of the heart through the superior and inferior vena cava. The blood then moves to the right ventricle where it is pumped into the pulmonary artery and capillary beds of the lungs. Here, oxygen is exchanged for CO_2 collected from the body tissues. The oxygen enriched blood then enters left atrium through the pulmonary vein. The flow of blood continues into the left ventricle where it leaves the heart via the aorta and enters systemic circulation, supplying the tissues of our body with oxygen and absorbing carbon dioxide through a complex capillary network. This network ends with the venous system which returns deoxygenated blood to the heart[39].

The vasculature consists of three types of vessels; arteries which carry oxygen rich blood from the heart, capillaries where gas, nutrient and waste exchange occurs and veins which return blood to the heart. Arteries and veins are similar in structure consisting of three layers; tunica intima, tunica media and tunica adventitia. The innermost tunica intima consists of an endothelium, a layer of simple squamous endothelial cells, with an associated basement membrane. Surrounding this layer is the tunica media made up of smooth muscle cells and elastic connective tissue and is responsible for vasodilation and vasoconstriction. The tunica adventitia encapsulates the tunica media in a layer of collagen, thereby acting as an anchor with the surrounding tissue[40].

The capillary network is structurally unique and allows for efficient exchange of materials. These vessels are the smallest of the vascular system and consist of a single layer of endothelial cells surrounded by a basement membrane. These vessels are the site at which angiogenesis typically occurs.

Steps of Angiogenesis

As stated previously, the vasculature is normally maintained in a quiescent state. In instances where pro-angiogenic stimuli predominate angiogenesis occurs through the induction of micro-vascular endothelial cells. In response to pro-angiogenic stimuli, such as VEGF, selective sprouting of endothelial cells from the existing vasculature is observed (Figure 2). This involves the disruption of cell-cell junctions, a reversal of cell polarity and increased protease expression, allowing for extracellular matrix degradation. This is followed by sprout expansion and directional chemotaxis. During this period, microvascular endothelial cells proliferate and migrate along pro- and inhibitory angiogenic gradients, achieving polarized extension of the sprout. As the sprout extends, extracellular matrix is deposited and pericytes are recruited to stabilize the forming vessel which fuses with the existing vasculature. Subsequent steps are poorly understood. Endothelial cell-cell interactions are maintained in the maturing vessel as vacuoles form and fuse first intracellularly and subsequently intercellularly, resulting in the formation of a primitive lumen. Following lumen formation blood flow is established. (41)

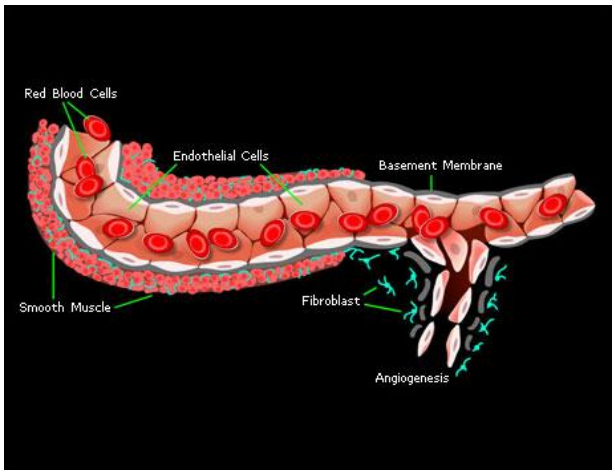

Figure 2. Angiogenic vessel sprouting. In response to pro-angiogenic signaling endothelial cells reverse polarity, secrete matrix degrading enzymes and begin to migrate along the stimulus gradient. As the newly formed vessel matures, basement membrane is layered down and associated cells such as vascular smooth muscle cells and fibroblast stabilize the growing structure (Genentech).

Thrombospondin - CD36 - Histidine Rich Glycoprotein Axis

Thrombospondin -1

Numerous reports have shown reduction or blockade of tumor growth by inhibition of angiogenesis. Thrombospondin-1(TSP) (Figure 3), in 1978, was the first endogenous anti-angiogenic molecule to be identified (42-44). The thrombospondin family consists of five extracellular calcium binding members (Thrombospondins 1-5) of which Thrombospondin-1 and 2 are most structurally similar (45, 46). TSP, like Thrombospondin 2, is a 450 kDa trimeric multi-domain matricellular glycoprotein.

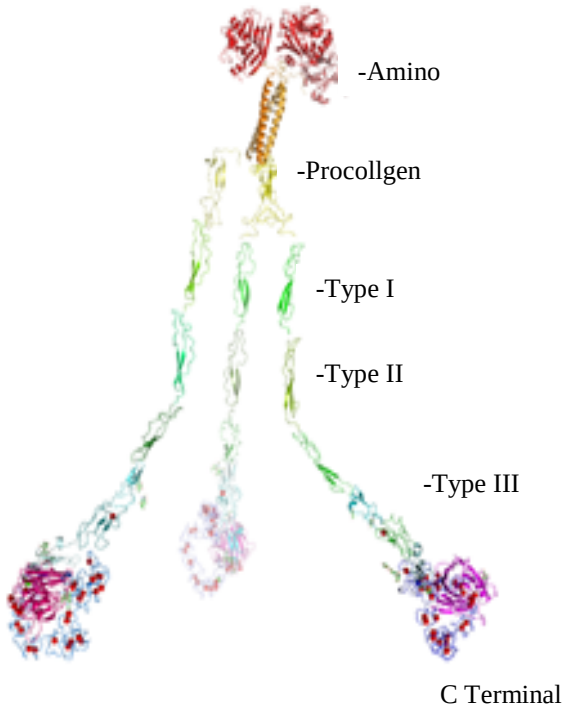

Figure 3. Thrombospondin 1 structure. Proposed crystal structure of Thrombospondin 1 trimer. From left; amino terminal domain, procollagen domain, type I repeats, type II repeats, type III repeats and carboxy terminal domain. (Mosher 2008)

Each monomer consists of, in order, an amino terminal heparin binding domain, procollagen homology domain, three properdin like type one repeats (TSRs), three epidermal growth factor like type 2 repeats, five calcium binding or type 3 repeats and a

lectin like carboxy terminal domain. Monomers are connected via disulfide bonds between the amino terminal and heparin binding domains[47]. TSP is expressed by several cell types including endothelial cells[48], smooth muscle cells[49], fibroblasts[50] and monocytes/macrophages[51] but contained primarily in platelet alpha granules, at a concentration of 82.6 ng/10^6 platelets, allowing for deposition at sites of injury or inflammation[52]. TSP may be soluble or cell bound, circulating in the plasma, at a concentration of 491 ng/ml[52], and incorporated into the extracellular matrix through interaction with fibrinogen[53], fibronectin[54], collagen[55], integrins[56] and heparin sulfate[57].

TSP has been implicated in diverse cellular functions and processes including synaptogenesis, inflammation/immune function, thrombosis and angiogenesis. The effects on synapse formation have been localized to the type 2 repeats of TSP, with null mice showing decreased synapse density[58,59]. Modulation of immune function and inflammation by TSP is complex, involving multiple cell types with differential effects in each. Activation of latent TGFβ by the RFK amino acid sequence of the TSRs of TSP, which Thrombospondin 2 lacks, is thought to influence monocyte/macrophage chemotaxis[60,61]. The process by which TSP activates latent TGFβ is currently unknown. Interaction of the C-terminal domain of TSP with membrane receptor CD47 on dendritic cells suppresses cytokine production, decreasing T-cell activation[62,63]. Additionally, direct interaction of TSP with T-cell integrin enhances adhesion and recruitment[64]. With regards to thrombosis, TSP has been shown to act as a bridge between platelets, binding surface integrins, thereby promoting aggregation[65]. Further, TSP has been shown to protect von Willebrand factor expressed by endothilal cells from matrix metalloprotease

cleavage at inflammatory sites thereby enhancing platelet recruitment[65]. These effects highlight the importance of TSP in tissue modeling and wound healing.

TSP is most well known as a vascular mediator. The role of TSP in vascular function centers around two concepts; control of vascular tone and regulation of angiogenesis. Control of vascular tone has been assessed by work by Isenberg and Roberts who have demonstrated nitric oxide (NO) modulation of vascular smooth muscle and endothelial cell function. NO effectively dilates vessels allowing for increased fluid extravasation into the surrounding tissues. Additionally, NO induces endothelial cell proliferation and migration. Two receptors, CD47 and CD36 have been implicated in vascular NO modulation by TSP[66]. CD47 was further shown to be necessary for TSP inhibition of NO signaling, with CD36 being only sufficient, therefore serving a possible supporting function[67]. More rigorous study is required to further delineate the TSP related functions of CD47 and CD36.

TSP is typically thought of as an angiogenic inhibitor, however groups have reported pro-angiogenic functions. Work by Aharonov showed a pro-angiogenic effect of TSP through the activation and recruitment of granulocytes in the rabbit cornea[68]. Additionally, *in vitro* rat aortic ring models showed recruitment of myofibroblasts by TSP, which promote angiogenesis through the secretion of heparin binding proteins[69]. These data do not necessarily conflict with the reported anti-angiogenic effect of TSP centered on microvascular endothelial cells as they focus on additional cell types.

The anti-angiogenic effects of TSP have been well characterized both *in vitro* and *in vivo*. TSP inhibits microvascular endothelial cell proliferation, migration and tube formation in response to basic fibroblast growth factor (bFGF) through the induction of

apoptosis[70,71]. Further, TSP inhibits vascularization in response to bFGF in corneal micropocket and subcutaneous matrigel assays[70,72]. Overexpression of TSP in the skin of transgenic mice results in decreased vascularization in full thickness skin wound healing models[73]. TSP overexpression in the skin has also been shown to inhibit angiogenesis and growth of squamous cell carcinoma tumors[74]. Conversely, TSP null mice display lengthened vascularization times in wound healing models and increased volume and vessel size in spontaneous breast cancer tumors[75,76].

From these studies, it appears TSP may function to both block and induce angiogenesis, possibly allowing for fine tuned modulation of vessel formation. Inhibition of angiogenesis in endothelial cells may be overcome by induction of pro-angiogenic stimuli by TSP in other cell types, i.e. granulocytes and myofibroblasts. This interplay and the role of TSP in pro-angiogenic induction require further study.

The anti-angiogenic effects of TSP have been localized to the TSRs. Treatment of microvascular endothelial cells with recombinant TSR induces apoptosis[77]. Additionally, TSR treatment effectively inhibits xenograft tumor growth and vascularization[77,78]. Each TSR assumes a unique 3 stranded anti-parallel barrel structure. The second and third strands form regular beta sheets, with the first strand possessing a novel "rippled" structure. The strand side chains of alternating Cysteine - Arginine - Tryptophan layers intercalate with one another and form the center of the structure. Exposed side chains of the arginine and tryptophan residues result in an overall positive charge on the binding face of this domain, which is recognized by the negatively charged binding site on CD36[79]. In addition to overall charge interaction between the TSRs and CD36, blocking antibodies and peptides have shown the amino acid sequence CSVTCG

of TSRs is vital to this interaction[80,81]. Work is currently under way by our group to solve the crystal structure of the TSR-CD36 interaction.

From the studies outlined for TSP we begin to appreciate its role in vascular functioning, i.e. inflammation, thrombosis, vessel dilation and angiogenesis. With regards to angiogenesis, several areas require further clarification. These include the angiogenic duality of TSP, role of CD36 in TSP function, both in normal and pathologic settings (i.e. tumor angiogenesis and formation), and evaluation of the overlapping roles of TSP receptors CD47 and CD36.

CD36

CD36, first described as glycoprotein IV, is a highly glycosylated 88 kDa class B scavenger receptor, referring to its 2 transmembrane domains, expressed on numerous vascular cell types including platelets, macrophages and microvascular endothelial cells (Figure 4)[82,83]. CD36 localizes to cholesterol-sphingolipid-rich rafts on the cellular membrane. These domains are known to be sites of signal transduction in eukaryotic cells[84].

CD36 recognizes ligands in a cell type specific manner, possibly due to differential co-receptor expression; CD9, α6β1, TLR2 and VEGFR2 in endothelial cells (85, 86, 87). Immunoprecipitation and immunofluorescent colocalization were utilized to show interaction. These studies have demonstrated modulation of TLR2 and VEGR2 signaling by CD36 and TSP. No evidence for modulation of the anti-angiogenic interaction of CD36 and TSP has been reported. With regards to endothelial cell biology, co-receptor expression presents a mechanism by which the inherent anti-angiogenic

potential of CD36 may be modulated. Further study is required to evaluate the role of these coreceptors in CD36 – TSP angiogenic functioning.

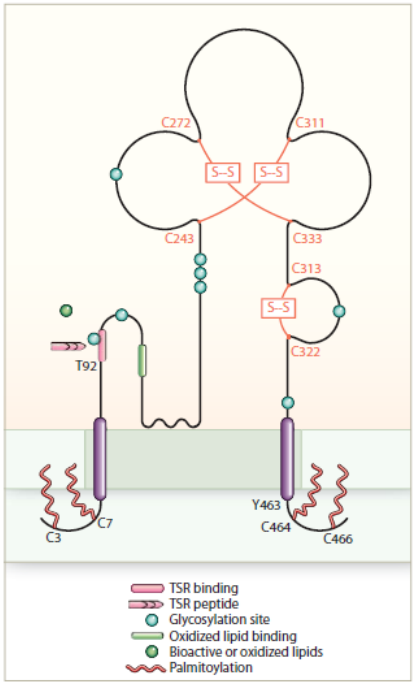

Figure 4. Membrane receptor CD36 structure. This receptor possesses two transmembrane domains, three disulfide linkages and multiple extracellular ligand binding domains. It localizes to lipid rich rafts on the cellular membrane (shaded). Modifications inc include intracellular palmitoylation and extracellular glycosylation (Silverstein 2009).

On platelets, CD36 interaction with several ligands, including oxidized low density lipoprotein lowers threshold activation. A recent study highlighted this effect showing correlation between human platelet CD36 expression and increased sensitivity to agonists, such as Adenosine diphosphate (ADP), resulting in increased aggregation and alpha granule release. Further, these affects are absent in mice null for CD36. As such, this study implicates CD36 in thrombosis and inflammation, serving as a potential marker for thrombotic risk[88].

On monocytes and macrophages, CD36 acts as a scavenger/ pattern recognition receptor mediating pathogen phagocytosis in which it recognizes lipid and lipoprotein moieties of bacterial origin[87]. This recognition involves a co-receptor function with toll

like receptor 2/6 heterodimer (TRL2/6). Additionally, CD36 binds and mediates the uptake of apoptotic cells and oxidized lipoproteins[89,90]. The former interaction has been implicated in macrophage foam cell formation and the progression of atherosclerotic plaques in murine models[91].

With regard to vascular endothelial cells the CLESH (CD36, LIMPII, *emp, SR-BI* Homology sequence 1) domain of membrane receptor CD36 has been shown to interact with the type I repeats (TSRs) of several proteins including Thrombospondins-1 and -2 and vasculostatin[81,92,93]. This domain is prototypic of the CD36 gene family which includes mammalian CD36, lysosomal integral membrane protein II (LIMPII), scavenger receptor class B-I and *Drosophila* epithelial membrane protein (*emp*)[94]. This family is defined by hydrophobicity, transmembrane domains with short cytoplasmic tails, an uncleaved N-terminal peptide sequence, a single extracellular domain and a C-terminal stop transfer domain[95]. Glutathione-S-Transferase (GST) fusion proteins were used to identify the CLESH domain in CD36, specifically amino acids 93-120, and subsequently in other family members, i.e. LIMPII[81,94]. Additional study is required to evaluate the physiologic importance of TSP interaction with all members of the CD36 gene family.

Interaction of the CLESH domain of CD36 with the TSRs of TSP initiates an anti-angiogenic signaling cascade in which CD36 interacts intracellularly with Src family tyrosine kinase $P59^{fyn}$ with down-stream signaling to P38 mitogen-activated protein kinase (MAPK) with accompanying caspase 3 like protease activation leading to induction of apoptosis in microvascular endothelial cells, thereby inhibiting angiogenesis[70]. This inhibition has been shown using in vitro and in vivo assays previously mentioned in this manuscript[70,72]. Additionally, TSP signaling leads to the

upregulation of tumor necrosis factor-related apoptosis-inducing ligand receptor (TRAIL-R), Fas ligand and tumor necrosis factor receptor 1[77,96,97]. Increases in TRAIL-R and Fas ligand were shown to be CD36 dependent.

With regards to pathologic angiogenesis, little is known with regards to the role of CD36. A recent study by Klenotic et.al. showed regulation of glioma tumor angiogenesis and growth by TSR containing protein vasculostatin in a CD36 dependent manner[25]. Direct regulation on endothelial cell migration was shown in vitro. Further, vasculostatin was shown to directly interact with the CLESH domain of CD36 through the use of GST fusion proteins. No evidence currently exists directly showing *in vivo* regulation of tumor angiogenesis and growth by the TSR-CD36 signaling axis.

From these studies, we gain a picture of a cell membrance receptor involved in numerous physiologic processes, primarily vascular in nature. Additionally, we highlight areas requiring further study, including role of coreceptor expression in CD36-TSP function, role of TSP binding to other CD36 gene family members and characterization of the role of CD36-TSP interaction in pathologic angiogenesis, i.e. tumor angiogenesis and progression.

HRGP

Histidine-rich Glycoprotein (HRGP), first described in 1972, is a 75 kDa soluble plasma protein produced by the liver, which circulates at relatively high concentrations; 100-150 ug/ml[98,99] (Figure 5). Additionally, HRGP is taken up by platelets and stored in the alpha granules[100]. It has been shown to interact with numerous ligands, including zinc, fibrinogen, vasculostatin, heparin, IgG and thrombospondin-1[98,101,102,103,104,105]. As such, HRGP has been implicated in diverse processes including immune function,

thrombosis and angiogenesis.

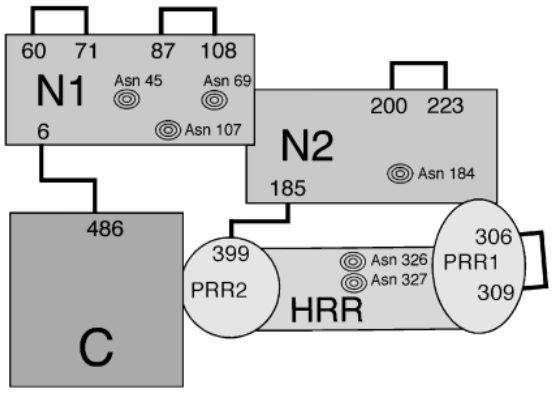

Figure 5. HRGP structure. HRGP consists of two N-terminal cystatin like domains, a histidine rich region flanked by two proline rich regions and a C-terminal domain. Disulfide linkages a shown by black lines and glycosylation sites by bull's-eyes (Parish 2005).

During normal immune response, antibodies recognize and complex with soluble antigen forming immune complexes which are subsequently cleared from the body. Inability to clear these complexes results in tissue deposition and the progression of pathologic conditions such as rheumatoid arthritis. *In vitro* data has shown that HRGP binds IgG and immune complexes, preventing insoluble immune complex formation, thereby implicating it in clearance and deposition[104,105,107]. Further, intact HRGP and fragments from the histidine rich region have been shown to possess anti-microbial and fungal activities, Enterococcus faecalis and Escherichia coli, and Candida albicans respectively[108,109]. This effect was mediated by HRGP binding of cell surface heparin with subsequent membrane destabilization[108]. *In vivo* modeling with HRGP null mice confirmed this effect[109]. Recent *in vitro* data has also implicated HRGP in the regulation of adhesion and spreading. Conflicting results have been reported, in which HRGP promotes or inhibits T cell adhesion[110,111]. Taken together these studies strongly support a role for HRGP in immune function.

HRGP has also been shown to be involved in thrombosis and fibrinolysis. As mentioned above, HRGP is able to bind heparin. This binding has been shown to inhibit the anti-thrombotic properties of heparin[112]. Further, HRGP binds fibrinogen, slowing the rate of degredation[102]. In contrast, HRGP has been shown to enhance plasminogen activation thereby speeding clot resolution[113]. Work utilizing HRGP null mice support the later role for HRGP with null mice forming and resoving clots more rapidly[114]. Thus, HRGP appears to promote clot formation and speeds its dissolution.

Aside from the thrombotic vascular function of HRGP, this protein has also been shown to exert pro- and anti-angiogenic activities. Two groups have reported anti-angiogenic functions by HRGP[115,116]. *In vitro* endothelial cell proliferation, migration and tube formation assays and *in vivo* chorioallantoic chick membrane and matrigel assays show inhibitory effects on angiogenesis. These studies make use of recombinant peptides or artificially generated proteolytic fragments not shown to be generated *in vivo*. Further they show localization of the anti-angiogenic properties of HRGP to the histidine rich region. These effects require release of the histidine rich region by a yet unknown mechanism. Plasmin presents as a possible candidate as it has previously been shown to cleave HRGP[117]. In addition to the proposed anti-angiogenic effects of a portion of HRGP, full length HRGP has been described as a pro-angiogenic molecule.

Work by our group has shown a pro-angiogenic role for HRGP mediated by a C-terminal CLESH homology domain. This domain, in similar fashion to the CLESH domain of CD36 binds the TSRs of several proteins including thrombospondin 1 and 2 and vasculostatin[92,103,118]. In vitro assays including endothelial cell proliferation, migration and tube formation show HRGP is able to inhibit the anti-angiogenic potential

of TSR containing proteins. Similarly, in vivo matrigel, corneal micropocket and tumor assays demonstrate HRGP inhibition of TSR inhibiton of angiogenesis. Tumor assays relied on overexpression of HRGP in tumor cells. No studies have assessed the role of ablation of HRGP in tumor angiogenesis and growth.

From these studies, we can appreciate the numerous roles of HRGP, most vascular in nature. We additionally gain a feel for areas requiring further study. HRGP appears to have a duality of angiogenic function whereby the intact molecule promotes angiogenesis, while cleaved fragments may inhibit. Further study is required to show generation of anti-angiogenic fragments in vivo and better clarify this duality. Additionally, further work is required to assess the role of this protein in pathologic angiogenesis, tumor growth and vascularization for example.

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CHAPTER II

ROLE OF CD36 – THROMBOSPONDIN - HISTIDINE-RICH GLYCOPROTEIN AXIS IN TUMOR ANGIOGENESIS AND GROWTH

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Abstract

The angiogenic switch in the development of cancer is an important therapeutic target. Work by our laboratory has established modulation of the interaction between CD36 and Thrombospondin 1 (TSP-1) by Histidine Rich Glycoprotein (HRG) in the regulation of angiogenesis. We have shown soluble HRG inhibits the anti-angiogenic potential of the CD36-TSP-1 pathway through a decoy receptor function whereby TSP-1 is bound and sequestered. The type I repeats (TSR) of TSP-1 were shown to mediate these interactions via a conserved domain in CD36 and HRG. We hypothesize the TSP-CD36-HRG axis regulates vascularization and growth in the tumor microenvironment. Lewis Lung Carcinoma (LL2) and B16F1 Melanoma tumor volumes were assessed in wild type (WT) and *hrg* or *cd36* null mice. LL2 tumor volumes were greater in *cd36* null mice and smaller in *hrg* null mice compared to WT. Immunofluorescent staining showed increased vascularity in *cd36* null vs. WT and WT vs. *hrg* null mice. No differences were

observed with B16F1. Western analysis showed increased expression of TSP-1 by LL2 vs B16F1 cells. Exogenous TSR expression in B16F1 cells restored effects similar to those obtained with LL2. These data suggest TSR-CD36 interaction leads to inhibition of angiogenesis in the tumor microenvironment and HRG modulates this interaction. Further, they suggest a mechanism by with insensitivity to TSR containing proteins may be achieved.

Introduction

Angiogenesis is the physiologic process by which new vessels sprout from the existing vasculature. In the normal adult setting, the vasculature is maintained in a quiescent state through a balance of angiogenic inhibitors, such as thrombospondin (TSP)-1, and inducers, such as vascular endothelial growth factor (VEGF). This balance between pro and anti- angiogenic stimuli is important in homeostasis, in particular in such conditions as pregnancy and wound healing. Loss of homeostatic balance resulting in excessive or insufficient angiogenesis has been implicated in numerous diseased states such as ulcerative colitis, diabetic retinopathy, obesity, psoriasis, rheumatoid arthritis, stroke, coronary artery disease and cancer[1].

It is well established that solid tumors will grow to 1-2mm by simple diffusion but require a blood supply in order to expand further and metastasize[2]. To this end tumors express pro-angiogenic substances such as basic fibroblast growth factor (bFGF) and VEGF which recruit blood vessels to the lesion through the induction of microvascular endothelial cell proliferation, migration and tube formation[3]. Previous studies have shown ablation of pro-angiogenic phenotypes by endothelial cell membrane receptor CD36[4,5]. CD36, an 88 kDa class B scavenger receptor, is expressed on numerous

vascular cell types including macrophages, platelets and microvascular endothelial cells. CD36 recognizes at least three classes of extracellular ligands – oxidized phospholipids, long chain fatty acids and proteins containing the so-called thrombospondin type I repeat (TSR)[6,7,8,9,10]. These receptor-ligand interactions mediate effects in a cell type specific manner. With regard to microvascular endothelial cells, a specific region of CD36 known as the CLESH domain interacts with high affinity with TSR domains of at least three endogenous anti-angiogenic proteins - thrombospondins-1 and -2 and vasculostatin[8,9,10]. These interactions initiate a complex intracellular signaling cascade involving the Src family tyrosine kinase $P59^{fyn}$ and p38 mitogen-activated protein kinase (MAPK) resulting in direct activation of caspase 3 like protease leading to induction of apoptosis[11]. Additionally, CD36 mediated cell death in microvascular endothelial cells has been reported to involve apoptotic receptors TNFR-1 and Fas[12,13]. These pro-apoptotic signals interrupt angiogenic responses induced by pro-angiogenic growth factors, such as bFGF and VEGF.

Despite abundant evidence in mouse models and human tumors that down-regulation of TSR-protein expression by genetic or epigenetic pathways in cancer cells promotes angiogenesis and thereby promotes tumor growth and metastasis, little is known whether modulating TSR interactions with its receptor, CD36, can influence tumor behavior[14,15,16,17]. In data described in this chapter we tested the hypothesis that genetic deletion of *cd36* or of *hrg*, a gene encoding a circulating CD36 decoy protein, would modulate tumor angiogenesis and tumor growth in syngeneic mouse tumor implantation models.

Histidine-Rich Glycoprotein (HRG) is a 75 kDa protein synthesized by

hepatocytes that circulates in plasma at relatively high concentrations (100-200 µg/ml)[18]. There are also abundant stores of HRG in the alpha granules of platelets (~371 ng/10^9 platelets) that can be released into specific microenvironments in response to platelet activation[19,20]. HRG is a modular protein that binds to proteoglycans, matrix proteins, divalent cations, and coagulation proteins. It possesses a domain analogous to the CLESH domain of CD36 that is able to bind TSRs of thrombospondin-1 and 2 and vasculostatin[8,9,10]. It is through this domain that HRG acts as a soluble decoy receptor for TSR domains, thereby blocking their binding to CD36 and regulating anti-angiogenic signaling on microvascular cells. As such, we hypothesized that tumors formed in mice lacking HRG will display increased CD36-TSP signaling resulting in decreased in vascularization and tumor growth.

In the present chapter we show that genetic deletion of *cd36* or *hrgp* in C57BL/6 mice effected tumor growth and vascularity. As predicted by our model, the effects were in opposite direction, with increased tumor growth in *cd36* null mice and decreased growth in *hrg* null mice. Also we show that these effects depended on tumor cell secretion of TSR-containing protein.

Methods

Materials

Mouse anti-VEGF receptor 2 antibody was from Cell Signaling Technology. Rabbit anti- VE-Cadherin and TSP polyclonal antibodies were from Abcam. Goat anti-rabbit IgG Alexafluor 488 conjugate and DAPI Prolong Anti-fade mounting media were from Invitrogen. Goat anti-rabbit horseradish peroxidase (HRP) was from Promega. Tissue Tek Optimal cutting temperature compound (OCT) was from Fisher Scientific.

Heparin, sucrose and paraformaldehyde were from Sigma.

Tumor Cells

Lewis Lung Carcinoma cells (LL/2) (CRL-1642) and B16F1 melanoma cells (CRL-6323) were obtained from the ATCC and maintained in Dulbecco's Modified Eagle Medium (Gibco) supplanted with 10% fetal bovine serum (Atlanta biologicals) and 0.5% penicillin/streptomycin (10,000U/ml, Gibco). Cells were incubated at 37˚C, 95% humidity and 5% CO2, grown in 75 cm^2 cell culture flasks (Corning) and passaged twice weekly. Cultures past 15 passages were not utilized. Stably transfected TSR-expressing B16F1 melanoma cell lines were generated by transfecting the cells with pSecTag2 secretory plasmid (Invitrogen) into which a cDNA encoding the TSR domains of mouse TSP-1 (amino acids 375-551) was cloned. Primers used for the cloning were ATATTGAAGCTTGCCCAGCGACTCTGCTGAC and ATATTGCTCGAGGTCCATCAATTGGGCAGTC. Transfection was done using the Fugene 6 reagent (Promega) as per manufacturors directions. Transfected clones were selected by antibiotic resistance using Zeocin (Invitrogen) at a concentration of 600 µg/ml. TSR expressing clones were identified by reverse transcription polymerase chain reaction and confirmed by western analysis of serum free cultured media.

Animals

All experiments and handling of mice were approved by the Institutional Animal Care and Use Committee (IACUC) of Cleveland Clinic. Mice were housed in a facility fully accredited by AALAC and in accordance with all federal and local regulations. All mouse strains used were of the same genetic background as the tumor cells - C57BL/6. Generation of *cd36* null and *hrg* null mice has previously been described[21,22]. Mice null

for *hrg* were initially of the 129/B6 background and were backcrossed 10 generations onto C57BL/6 background.

TSP-1 and TSR Expression Analysis

Secretion of TSP-1 or recombinant TSR peptide by mouse tumor cells was assessed after culture in serum free media for 48 hours. Post culture media was collected and proteins precipitated with trichloroacetic acid. Precipitated samples were washed twice with acetone, resuspended in laemmli sample buffer and then electrophoresed on SDS-PAGE (10%) gels under reducing conditions. Proteins were transferred on to polyvinylidene fluoride (PVDF) at 250ma for 3 hours at 4C. Membranes were blocked with 5% milk in 0.1% triton tris buffered saline (TBS). Primary anti-TSP and secondary anti-rabbit HRP antibodies were utilized at 1:1000 dilutions. Blots were developed using the ECL Plus system (Fisher). Purified TSP 1 and HRGP were used as controls.

Syngeneic Tumor Implantation Studies

C57Bl/6, *cd36* null or *hrgp* null mice were anesthetized with ketamine and xylazine (IP, 50 mg/kg ketamine, 5 mg/kg xylazine). Lewis Lung Carcinoma (LL2), B16F1 Melanoma or TSR-transfected B16F1 Melanoma cells were injected subcutaneously onto the backs of eight week old male animals at a concentration of 50,000 cells/50 µl. Tumor volumes were assessed over 17 days using a standard formula ($V = L \times W^2 \times 0.52$), which assumes a hemi elliptical shape. Mice were anesthetized at each time point. Following terminal measurement, mice were euthanized by CO_2 and perfused with heparin (10 U/ml) and 4% paraformaldehyde. Tumors were then resected, incubated overnight in 15% sucrose and embedded in OCT. Samples were sectioned at a thickness of 10µm. Overall cellularity and structure were evaluated by hematoxylin and

eosin (H&E) staining. Blood vessel density was assessed by immunofluorescent staining using anti-VEGFR2 or VE-Cadherin antibodies. Average vessel count/mm^2 was calculated from 6 fields of view per tumor taken at 200x magnification using a Leica DM5500B automated upright microscope system.

Statistics

Power calculations were performed *priori* to determine group size using a standard formula, $n=2[(u_a + u_b)s/d]^2$, assuming variance of 20%, confidence of 95%, beta error of 0.1 and standard error of 10%. Optimal group size was calculated to be 7 individuals. Differences between groups were calculated by Student's unpaired T-test. Outlying values were excluded using Grubb's outlier test.

Results

Syngeneic Lewis Lung Tumors in *Cd36* Null Mice Were Larger and More Vascular Than in Wildtype

LL2 cells when injected into mice lacking *cd36* produced tumors of greater size than those injected into age and sex matched wildtype mice (Figure 6).

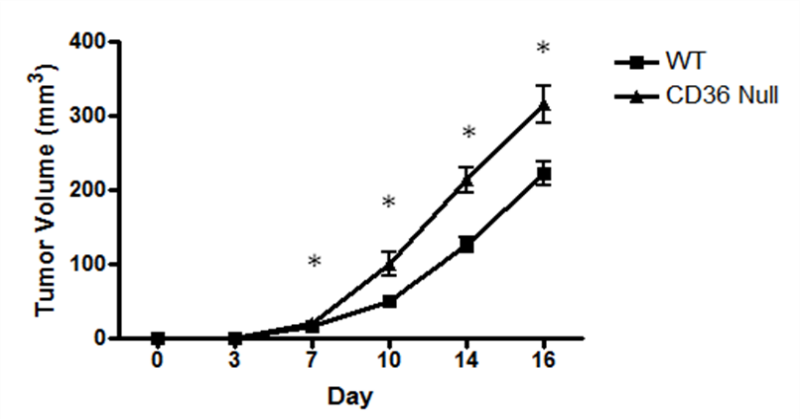


Figure 6. Cd36 deletion in mice enhances syngeneic tumor growth. Lewis Lung carcinoma cells (A) or B16F1 melanoma cells

These differences were statistically significant ($P<0.05$) at all time points at which tumor volumes were measurable. Mean tumor volumes in *cd36* null vs wildtype mice were 21.0 mm^3 vs 15.8 mm^3 at day 7, 100.4 mm^3 vs 52.1 mm^3 at day 10, 213.6 mm^3 vs 136.3 mm^3 at day 14 and 316.2 mm^3 vs 237.7 mm^3 at day 17 respectively. Tumors formed in *cd36* null animals displayed increased areas of necrosis as evidenced by H&E staining (data not shown) and greater vascularization (Figure 7). On average *cd36* null tumors contained 17.0 vessels/mm^2 vs 12.2 vessels/mm^2 in wildtype ($P<0.05$). These data are consistent with our hypothesis that CD36 mediates an anti-angiogenic phenotype resulting in decreased tumor vascularization and growth.

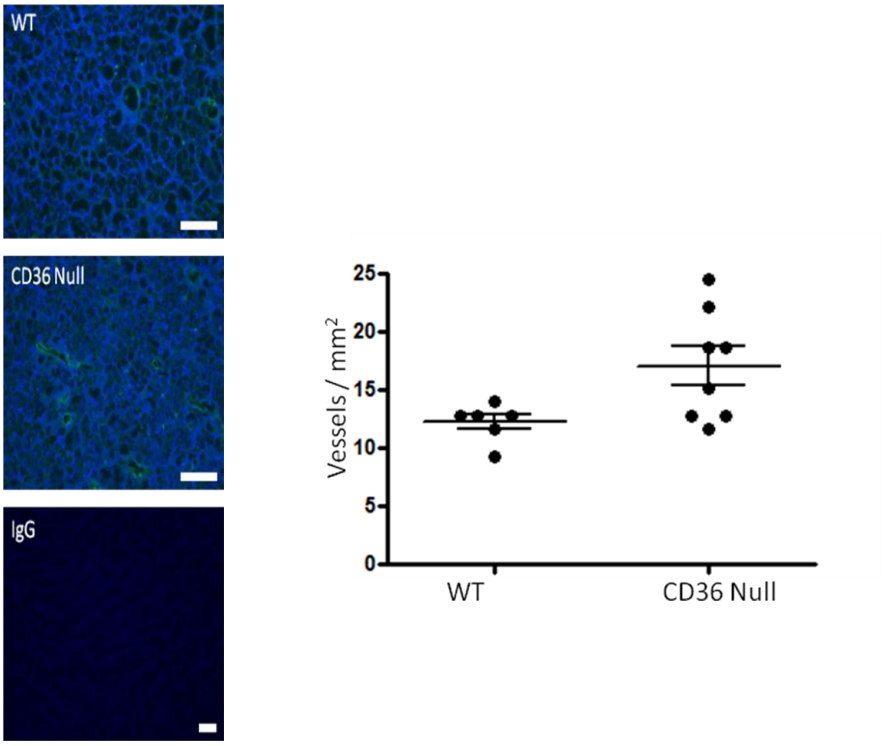


Figure 7. Cd36 deletion in mice enhances Lewis Lung tumor vascularity. (A) Lewis Lung tumors as in Figures 6 were dissected, sectioned and examined by immunofluorescence microscopy using anti-VEGF receptor antibody (green) to detect blood vessels. DAPI stained nuclei are blue. Magnification bars represent 100µm. IgG control is shown in bottom panel as negative control. (B) Vessel densities measured as vessels per mm^2

Syngeneic Lewis Lung Tumors in *hrg* null mice were smaller and less vascular than in wildtype

When injected into mice lacking *hrg*, LL/2 tumors were smaller and less vascular compared to those in wildtype mice (Figure 3A). Average tumor volume in *hrg* null vs wildtype individuals were 10.4 mm^3 vs 20.0 mm^3 at day 7, 33.9 mm^3 vs 49.1 mm^3 at day 10, 93.9 mm^3 vs 126.7 mm^3 at day 13 and 189.6 mm^3 vs 316.1 mm^3 at day 17 respectively. Differences at all points day 7 and beyond were significant at $P<0.05$. Tumors in *hrg* null mice displayed less necrosis (data not shown) and were characterized by decreased vasculature compared with wildtype (Figure 4); on average *hrg* null tumors contained 7.1 vessels/mm^2 vs 13.6 vessels/mm^2 in wildtype ($P<0.05$). These data are consistent with our hypothesis that HRG modulates CD36-TSR anti-angiogenic signaling.

Tumor cell TSR expression is required for regulation of syngeneic tumor growth and vascularity by genetic manipulation of *cd36* or *hrg*

In sharp contrast to the results seen with LL2 cells, implantation of B16F1 melanoma cells resulted in tumors of similar size (Figure 8 and 9) and vascularity (not

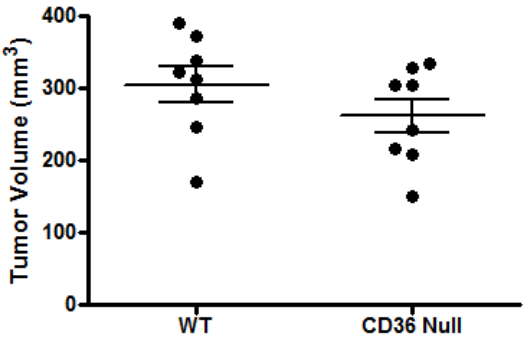


Figure 8. Cd36 deletion in mice enhances syngeneic tumor growth. Lewis Lung carcinoma cells were injected in the backs of cd36 null or wild type C57BL/6 mice (50,000 cells/mouse). Tumor volumes were assessed over 17 days following implantation. *P<0.05.

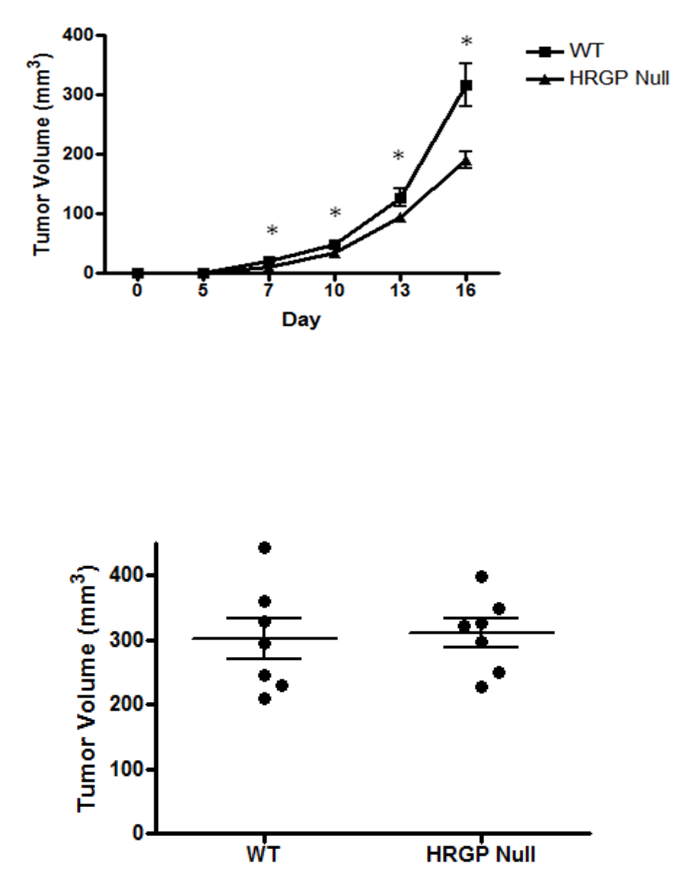


Figure 9. Hrg deletion in mice suppresses syngeneic tumor growth. Lewis Lung carcinoma cells (A) or B16F1 melanoma cells (B) were injected in the backs of hrg null or wild type C57BL/6 mice (50,000 cells/mouse). Tumor volumes were assessed over 18 days following implantation. *P<0.05.

shown) regardless of genetic background of the host. We hypothesized that these differences may relate to differing levels of TSR protein expression and indeed immunoblot analysis of conditioned media from the tumor cell lines showed readily detectable TSP-1 in the postculture media from LL2 cells, but not from B16F1 cells (Figure 10A). We therefore generated stably transfected B16F1 cell lines that expressed and secreted recombinant TSP-1 TSR domains. As shown in Figure 10B, Clone 11 expressed abundant TSR and was used for all further experiments. TSR transfection restored responsiveness to the CD36/HRGP system. TSR expressing B16F1 cells

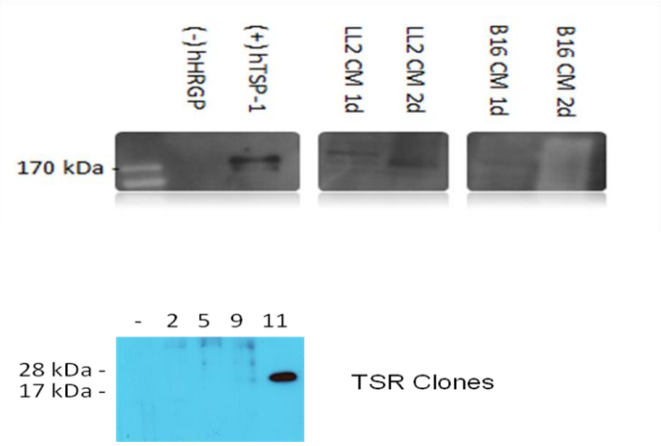
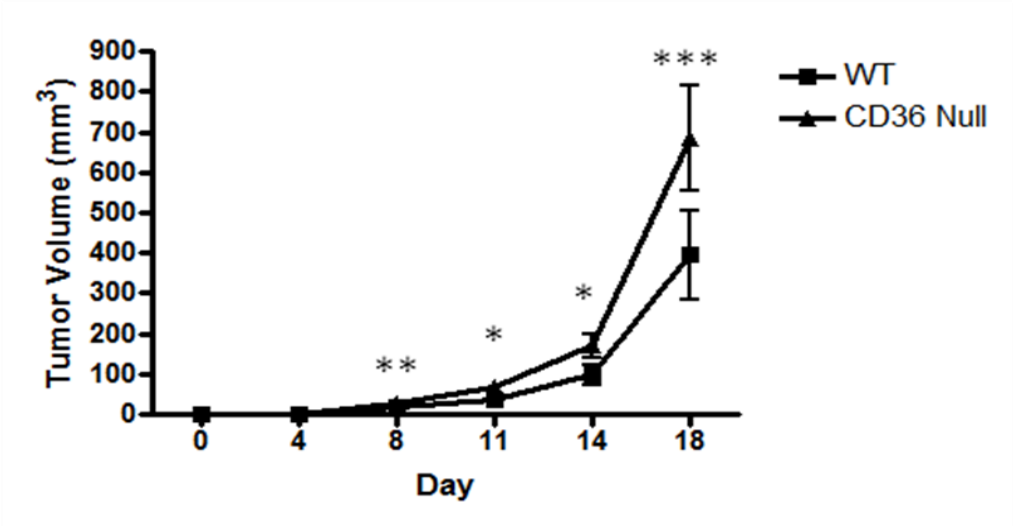


Figure 10. Thrombospondin-1 secretion from cultured Lewis Lung and B16F1 melanoma cells. (A) Lewis Lung (LL2) or B16F melanoma cells were cultured in serum free media for 24 hours (1d) at which point proteins in post culture media (CM) were precipitated by TCA, separated under reducing conditions by SDS/PAGE and analyzed by immunoblot using anti-TSP-1 antibody. TSP-1 monomers were detected at 170 kDa in the media conditioned by LL2 cells, but not B16F1 cells. Purified human HRG and TSP were used as controls. (B) Conditioned media was collected from 4 different antibiotic resistant clones of TSR transfected B16F melanoma cells and analyzed by immunoblot as in panel A. Clone 11 expressed abundant anti-TSP reactive material at the appropriate molecular weight of recombinant TSR and was utilized for subsequent tumor studies.

produced larger and more vascular tumors in *cd36* null mice (Figures 11A, 7A left and 7B top) and smaller and less vascular tumors in *hrgp* null mice (Figures 11B, 7A right and 7B bottom) when compared to age and sex matched wildtype controls. Average tumor volume in *cd36* null vs wildtype individuals were 27.8 mm^3 vs 17.5 mm^3 at day 8 ($P=0.08$); 67.7 mm^3 vs 37.8 mm^3 at day 11 ($P<0.05$); 170.5 mm^3 vs 98.0 mm^3 at day 14 ($P<0.05$); and 685.1 mm^3 vs 394.7 mm^3 at day 18 ($p=0.06$). On average *cd36* null tumors contained 16.3 vessels/mm^2 vs 9.1 vessels/mm^2 in wildtype ($P<0.05$). In the *hrgp* null mice the average tumor volumes compared to wildtype were 5.8 mm^3 vs 53.2 mm^3 at day 11; 87.9 mm^3 vs 255.3 mm^3 at day 15; and 211.0 mm^3 vs 651.7 mm^3 at day 18. All of these differences were significant at $P<0.05$. The tumors formed in *hrg* null animals were more vascularized with on average 5.4 vessels/mm^2 vs 10.1 vessels/mm^2 in wildtype ($P<0.05$). These data further support our hypothesis that CD36-TSR interaction mediates an anti-angiogenic phenotype with modulation by HRG.



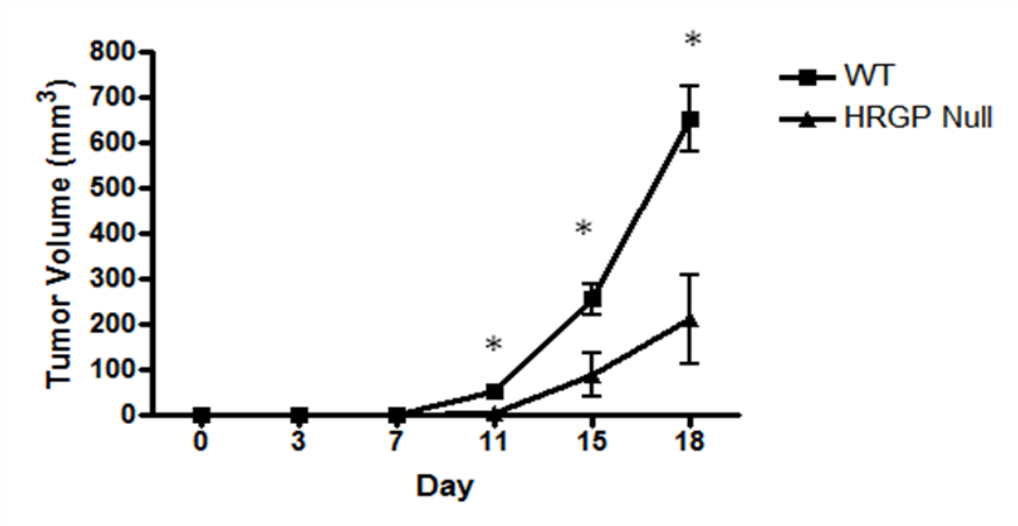


Figure 11. TSR transfected B16F1 melanoma cells show enhanced tumor growth in cd36 null mice and suppressed tumor growth in hrgp null mice. 50,000 cells from a stably transfected B16f1 melanoma cell line (Clone 11) were injected in the backs of cd36 null (A) or hrgp null (B) mice. C57Bl/6 mice were used as controls. Tumor volumes were assessed at timed points as in Figures 1 and 3. *P<0.05; **P=0.08; ***P=0.06.

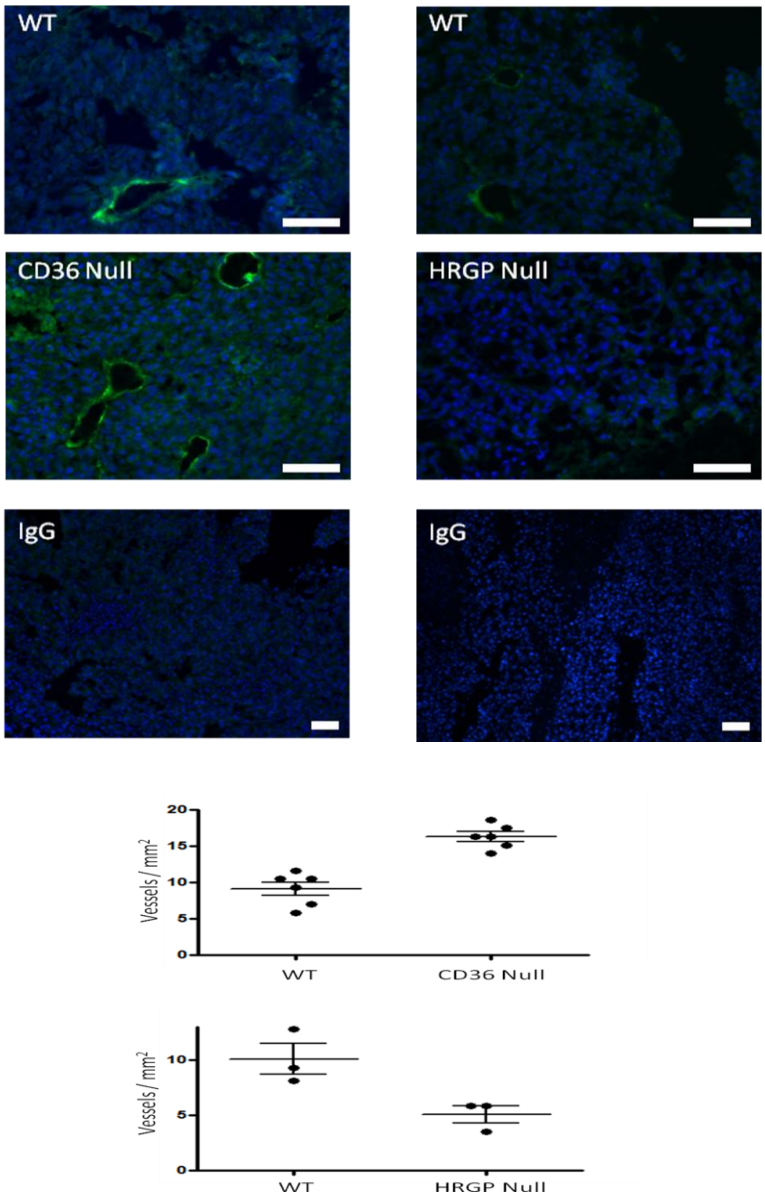


Figure 12. TSR transfected B16F1 melanoma cells show enhanced tumor vascularity in cd36 null mice and suppressed tumor vascularity in hrgp null mice. (A) Tumors from TSR transfected B16F1 melanoma cells as in Figure 6 were dissected, sectioned and examined by immunofluorescence microscopy using anti-VE-Cadherin antibody (green) to detect blood vessels. DAPI stained nuclei are blue. Magnification bars represent 100µm. IgG control is shown in bottom panels as negative control. (B) Vessel densities measured as vessels per mm2.

CD36- TSR signaling inhibits microvascular endothelial cell migration, proliferation and tube formation in *in vitro* and *in vivo* models. Our group has shown that this important endogenous anti-angiogenic system can be dampened by HRG, a protein

with structural homology to CD36 that acts as a decoy for TSR. *In vitro* assays of microvascular endothelial cell migration, proliferation, and tube formation; and *in vivo* assays of angiogenesis in mouse corneal micropockets and implanted matrigel showed that addition of exogenous HRG blocks TSP-1, TSP-2 and vasculostatin binding to CD36 and thereby inhibits TSR-mediated vascular cell responses[9,10,23]. HRG circulates in high concentrations, can be released from activated platelets, and accumulates in perivascular matrix; thus it is "poised" to serve an important role in regulating microvascular CD36-TSR signaling *in vivo*[24,25]. This may have particular relevance to tumor angiogenesis since HRG has been shown localize in the stromal connective tissue of human tumors, including breast cancer and glioblastoma, and to mask the TSR domain of TSP[10,23]. The potential importance of this system in carcinogenesis is supported by abundant data showing that *TSP1* has potent tumor suppressor activity and that genetic or epigenetic down-regulation of TSP-1 expression is associated with progression of numerous human cancers and enhanced tumor angiogenesis. We thus hypothesized that accumulation of HRG in the tumor microenvironment would promote tumor growth, similar to loss of tumor cell TSR expression, and that down-regulation of the receptor, CD36 would have an opposite effect.

In the experiments described in this manuscript we used mouse genetic models to provide direct evidence in support of this hypothesis. In the absence of CD36, transplanted syngeneic tumors were larger and displayed increased vascularity, while in the absence of HRG, tumors were smaller and displayed less vascularity. Importantly, these host-mediated effects required production and secretion of TSR-containing protein by the transplanted tumor. TSP-1 secreting Lewis Lung Cancer cells were sensitive to

loss of CD36 or HRG, while TSP-1 negative B16F1 melanoma cells were not sensitive unless they were stably transfected with a TSR-expressing plasmid. Our data suggest that tumor cells could induce a state of functional TSR deficiency and hence promote angiogenesis and tumor growth) by remodeling their micro-environment to down-regulate microvascular CD36 expression and/or increase accumulation of HRGP (discussed below). In regard to the former, we recently found that lysophosphatidic acid (LPA) activates a protein kinase D-mediated signaling pathway in microvascular endothelial cells that transcriptionally silences *cd36* and thereby promotes angiogenesis[26]. Since both tumor cells and inflammatory cells are potential sources of LPA, this could be highly relevant to tumor biology.

HRG accumulation in tumor microenvironments would be expected to relate to at least two processes known to promote tumor growth and metastasis – VEGF expression and platelet activation[27,28]. In addition to promoting angiogenesis, VEGF is a potent microvascular permeability factor that contributes to the "leaky" vasculature of tumor beds[29]. In this milieu, plasma proteins such as HRG escape from the confines of the vessel and permeate into the tumor bed. Similarly, platelet-tumor cell interactions have been studied for many years and are known to promote both tumor growth and thrombosis[30]. Platelet accumulation and activation in a tumor microenvironment would have many effects, including release of both TSP-1 and HRG.

HRG was first characterized in 1978 as a molecule which bound heme and certain metal ions[31]. Today, it is viewed as an adapter protein due to its multi-domain nature and multiple ligand binding capacity, and has been implicated in diverse functions including immunity, thrombosis, cell adhesion and angiogenesis[32-34]. The potential to develop

HRG as a novel therapeutic target to regulate angiogenesis is complicated by reports from two groups showing in contrast to our work, that HRG has anti-angiogenic activity[35,36]. The mechanism for this activity has not been defined, but it is mediated by the histidine-proline rich region of the protein. Our genetic models and abundant *in vitro* and *in vivo* studies using intact, native HRG strongly support a pro-angiogenic role for HRG in the presence of TSR proteins and did not show any anti-angiogenic activity, even in the absence of TSR proteins. The most likely explanation for this apparent controversy is that the anti-angiogenic activity requires proteolytic release of the histidine/proline-rich domain. Whether there is an endogenous pathway to release the domain has not been demonstrated, but precedent exists for proteolytic peptide fragments having opposite biological activity than their "parent" protein[37].

In summary, we showed in these studies that modulating tumor cell expression of TSR proteins or expression in the non-transformed tumor microenvironment of CD36 or HRG had significant impact on tumor angiogenesis and tumor growth. Numerous pro- and anti-angiogenic therapies are in clinical trials, among them ABT-510 and ABT-898, which are peptide mimetics of the TSR domain of TSP-1. These compounds have shown potential for treatment of cancer suggesting that targeting CD36 or HRG could present effective alternative approaches to enhance or inhibit TSR action[38-40].

Acknowledgements

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CHAPTER III

ROLE OF ZINC IN A DISINTEGRIN AND METALLOPROTEINASE WITH THROMBOSPONDIN MOTIFS 1 (ADAMTS1) PROCESSING OF THROMBOSPONDIN 1

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Abstract

Thrombospodin 1 (TSP) is a well characterized vascular regulating protein. The anti-angiogenic effects of TSP have been localized to the type I repeats (TSRs). Additionally, vasodilatory and dendritic cell mediated T cell modulatory effects have been localized to the C-terminal domain. Previous work has demonstrated TSP cleavage by ADAMTS1, with release of anti-angiogenic peptides. We propose zinc mediated modulation of TSP cleavage by ADAMTS1. Upon addition of zinc to the system, an additional TSP cleavage site is generated, from those previously described. This site is located in the C-terminal domain of TSP, thereby presenting a method of regulating CD47 interaction. Regulation of this interaction may have implications in vasodilation, angiogenesis and immune function regulation.

Introduction

Remodeling of the extracellular matrix by matrix metalloproteases is vital to numerous homeostatic processes including angiogenesis. The ADAMTS (a disintegrin and metalloproteinase with thrombospondin motif) family of extracellular, zinc dependent proteases consists of 19 members, all of which contain the type I repeats (TSRs) of Thrombospondin I (TSP)[1]. Theses enzymes are involved in collagen processing, cleavage of matrix proteoglycans, thrombosis and inhibition of angiogenesis[2].

With regard to inhibition of angiogenesis, ADAMTS 1 and 8 have been shown to inhibit vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) induced endothelial cell proliferation *in vitro* and angiogenesis in the *in vivo* corneal micropocket and chick chrioallantoic membrane assays[3]. ADAMTS1 mediates these effects via binding bFGF and VEGF[4,5]. Additionally, work by Dr. Arispe has demonstrated that ADAMTS1 may cleave thrombospondin 1 and 2 in such a manner that protein releases three monomers each containing the type one repeats[6]. The proteolytic cleavage of thrombospondin 1 and 2 is proposed as a mechanism by which the anti-angiogenic potency of matrix bound thrombospondin may be modulated. Here after we will focus on TSP, however it should be noted that many of the anti-angiogenic properties cited are shared with thrombospondin 2 as both proteins are structurally similar, containing the type I repeats.

TSP is a 450 kDa trimeric multidomain matricellular glycoprotein. It was the first endogenous anti-angiogenic molecule identified. TSP, *in vitro*, inhibits microvascular endothelial cell proliferation, migration and tube formation in response to basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF), through the

induction of apoptosis[7,8]. Further, TSP inhibits vascularization, *in vivo*, in response to pro-angiogenic stimulus as demonstrated by corneal micropocket and subcutaneous matrigel assays[7,9].

The anti-angiogenic effects of TSP have been localized to the type I repeats (TSRs). *In vitro* treatment of microvascular endothelial cells with recombinant TSR has been shown to induce apoptosis[10]. Additionally, TSR treatment, *in vivo*, effectively inhibits tumor growth and vascularization[10,11].

In addition to the anti-angiogenic role localized to the TSRs, TSP also mediates vascular biology via its C-terminal domain. Interaction of the C-terminal domain of TSP with membrane receptor CD47 on dendritic cells suppresses cytokine production, decreasing T-cell activation[12,13]. CD47 was further shown to be necessary for TSP inhibition of NO signaling, which mediates vessels dilation via vascular smooth muscle cells, allowing for increased fluid extravasation into the surrounding tissues[14].

Previous study of the cleavage of TSP by ADAMTS1 has excluded an important heavy metal, zinc, in the regulation of protease function. We hypothesize zinc modulates ADAMTS processing of TSP. To this end we utilized an in vitro physiologic system in the presence and absence of zinc to assess the role off this heavy metal. We show that addition of zinc induces additional processing of TSP at the C-terminal domain.

Materials

Thrombospondin Isolation

One unit, 250 cc, of outdated concentrated platelets was obtained from the Cleveland Clinic Blood Bank. Samples were spun at 200 g to remove contaminating red blood cells. Platelets were then pelleted at 900 g and resuspended in Broekmanns buffer,

repeated 3 times to wash. Pellets were combined and resuspended in a final volume of 15ml 20mM Tris 150mM NaCl pH 7.6 buffer and Calcium added to a final concentration of 2mM. Platelets were stimulated with 3 U/ml thrombin, followed by hirudin (6 U/ml) inhibition. Samples were then spun at 20,000 g and supernatant applied to a 1 ml heparin column. TSP was eluted at 0.45M NaCl. Elution fractions were run on denaturing gels and positive fractions combined and diluted 1:3 in 20mM Tris, pH 7.4. Sample was then applied to a 1 ml mono Q anion exchange column and TSP was eluted over 0-0.8M NaCl gradient (~0.6M). Elution fractions were run on denaturing gels and positive fractions quantified for protein concentration.

ADAMTS1 digestion of Thrombospondin

Standard digestions were carried out under physiologic conditions in pH 7.4 buffer containing 20mM Tris, 150 mM NaCl, 10mM CaCl and 15 µM ZnCl. Following optimization, 2µg purified TSP was digested with 400ng recombinant ADAMTS1 (R&D Systems) overnight at 37C, a 1:5 ratio. Calcium and Zinc were chelated from digestion buffers with EGTA and Zincon respectively. Following digestion, samples were run on 10% reducing gels and coomassie stained.

Sequence Analysis

Sequence analysis was carried out by the Mass Spectroscopy Core of the Cleveland Clinic Foundation. Proteins were excised from reducing poly-acrylamide gels and destained in 50% ethanol, 5% acetic acid. Excised bands were the reduced with dithiothreitol, iodoacetamide alkylated and trypsin digested overnight. Resulting peptides were extracted and dried to less than 30 µl. Liquid chromatography-mass spectroscopy was performed using the Thermofisher LTQ ion trap mass spectrometer

with a 8cm x 75 µm id Phenomenex Jupiter C18 reverse phase capillary chromatography column. Peptide samples were inected using an Eksigent nanoflow liquid chromatography system and eluted with an acetonitrile-0.05M acetic acid gradient. Generated collisionally induced dissociation (CID) spectra were used to perform Mascot NCBI database searches.

Results

ADAMTS1, under physiologic conditions cleaves TSP N-terminally, with decreases in detectable peptides prior to amino acid 313 (Figure 13, 14-5). This site is located in the disulfide linker region (Figure 15). Addition of zinc to digestion buffers resulted in more efficient N-terminal cleavage and the generation of an additional

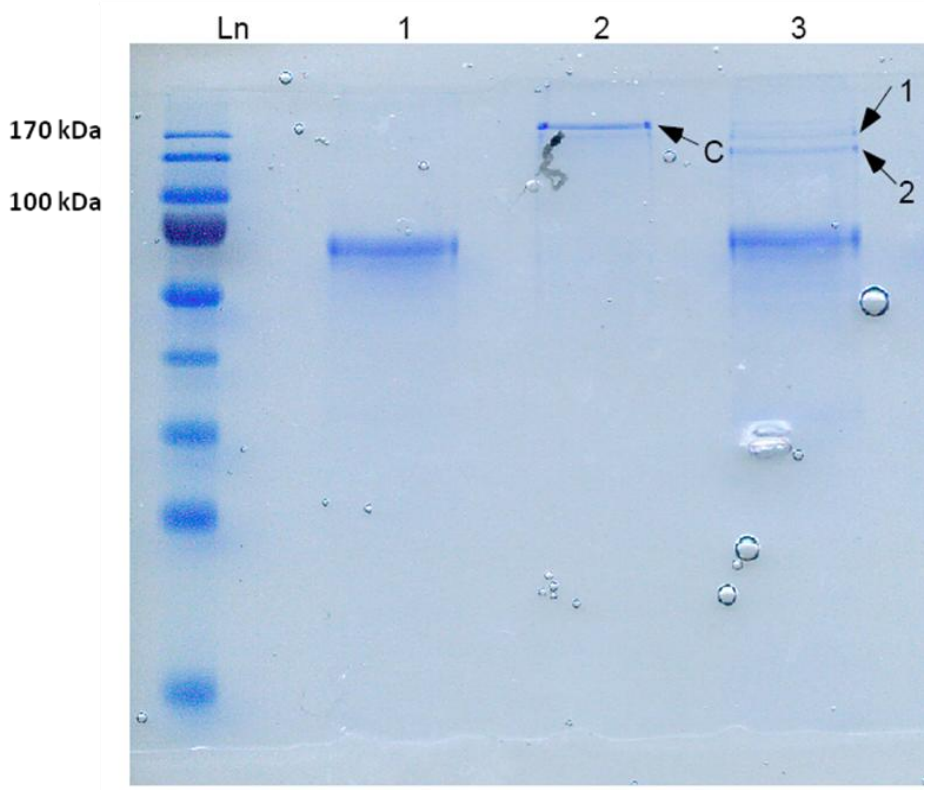

Figure 13. ADAMTS1 generates multiple fragments of Thrombospondin 1. ADAMTS1 alone (lane 1), Thrombospondin 1 alone (lane 2), Thrombospondin cleavage by ADAMTS1 (lane 3). In physiologic buffer conditions ADAMTS1 processing of Thrombospondin 1 yields 2 fragments (1 & 2, lane 3). Fragment 1 corresponds to a N-terminally truncated form of Thrombospondin 1, prior to amino acid 313. Fragment 2 corresponds to a C-terminally truncated form of Thrombospondin 1, after amino acid 1054.

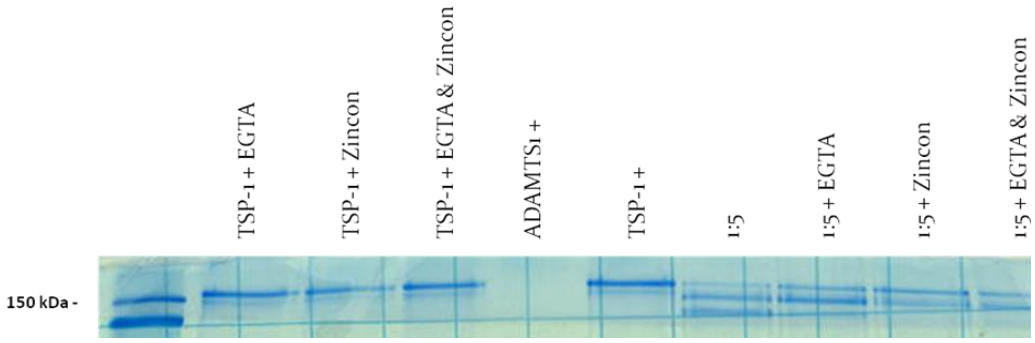

Figure 14. ADAMTS1 utilizes calcium and zinc in Thrombospondin 1 cleavage. ADAMTS1 requires zinc and to a lesser extent calcium in the generation TSP fragments. Requirement for metal ions, to agreater degree zinc, is greater for C-terminal cleavage of TSP. EGTA and Zincon were used to chelate calcium and zinc respectively from the digestion buffers. Lane 1 TSP with EGTA alone, Lane 2 TSP with Zincon alone, Lane 3 TSP with EGTA and Zincon, Lane 4 ADAMTS1 alone, Lane 5 TSP alone, Lane 6 TSP digestion by ADAMTS1 at 1:5 ratio, Lane 7 TSP digestion with EGTA, Lane 8 TSP digestion with Zincon, Lane 9 TSP digestion with EGTA and Zincon.

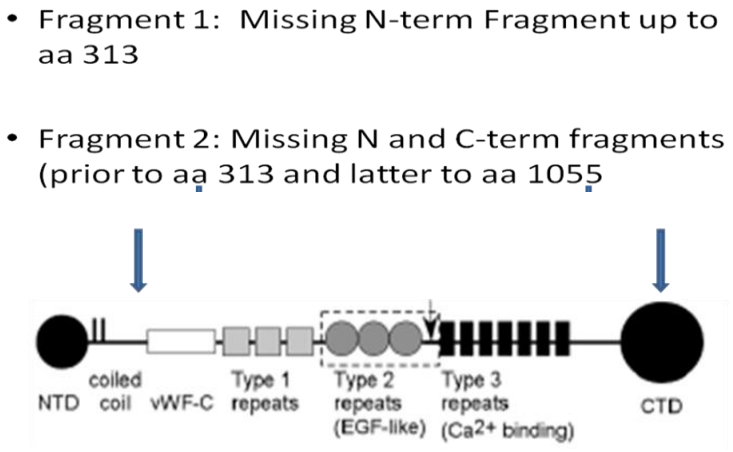

Figure 15. ADAMTS1 generated fragments of Thrombospondin 1. The ADAMTS1 cleavage sites, indicated by blue arrows, on an intact monomer of Thrombospondin 1. Cleavage site 1 is located in the disulfide linker region. Cleavage site 2 is located in the globular C-terminal domain.

fragment (Figure 13, 14). This fragment corresponds to a C-terminal cleavage after amino acid 1054, as identified by decrease peptide detection (Figure 16, 17). This site is located within the globular C-terminal domain of TSP (Figure 15). Chelation of calcium reduced N-terminal and C-terminal cleavage of TSP by ADAMTS1. Chelation of zinc reduced N-terminal cleavage and abolished C-terminal cleavage of TSP by ADAMTS1 (Figure 14).

$[M+H]^+$	Sequence	Amino Acid	Full	Band 1	Band 2
2195	IPESGGDNSVFDIFELTGAAR	21-41	+	-	-
1030	GPDPSSPAFR	51-60	+	+	-
2579	IEDANLIPPVPDDKFQDLVDAVR	61-83	+	+	-
989	GFLLLASLR	87-95	+	+	-
1208	SITLFVQEDR	155-164	+	+	-
1395	FVFGTTPEDILR	217-228	+	+	-
1247	TIVTTLQDSIR	289-299	+	+	-
1660	RPPLCYHNGVQYR	314-326	+	+	+
1596	QVTQSYWDTNPTR	1042-1054	+	+	+
1551	NALWHTGNTPGQVR	1078-1091	+	+	-
924	TLWHDPR	1092-1098	+	+	-

Figure 16. ADAMTS1 produces N and C terminal cleavage of Thrombospondin 1. Molecular weight ([M+H]+), peptide sequence and amino acid positions are given from left to right. Mass spec analysis identified multiple peptide matches to Thrombospondin 1 in the full length protein, 11 shown above (column full). ADAMTS1 cleavage of TSP in the presence of calcium (Band 1) resulted in N-terminal cleavage with complete absence of a small N-terminal peptide (amino acids 21-41). ADAMTS1 cleavage of TSP in the presence of calcium and zinc (Band 2) resulted in more efficient N-terminal cleavage with absence of N-terminal peptides up to amino acid 313. Additionally, addition of zinc to the digestion buffer induced C-terminal cleavage of TSP with peptides missing after amino acid 1054.

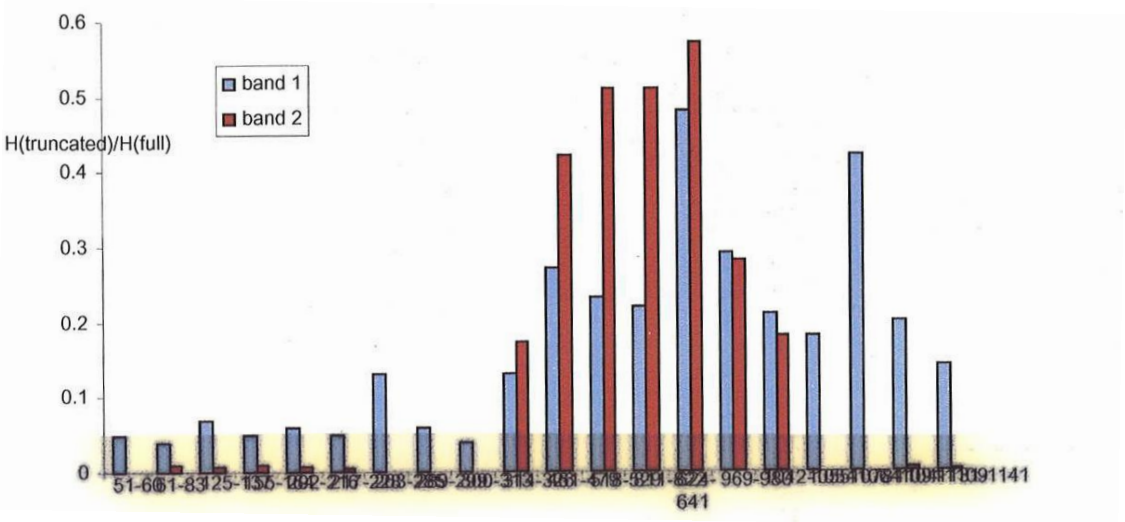

Figure 17. ADAMTS1 N and C terminally cleaves Thrombospondin 1. More sensitive quantification of identified peptides are shown normalized to full length TSP (H(truncated)/H(full)). All peptides were detected in each digestion sample, possibly owing to carry over contamination as the same column was used. ADAMTS1 cleavage of TSP in the presence of calcium (Band 1) resulted in reduction of N-terminal peptides prior to amino acid 313 as compared to abundance in full length TSP. ADAMTS1 cleavage of TSP in the presence of calcium and zinc (Band 2) resulted in increased reduction of N-terminal peptides prior to amino acid 313 and after amino acid 1054 as compared to full length TSP.

Discussion

ADAMTS1 has previously been shown to inhibit angiogenesis *in vitro* and *in vivo*[3,4]. Work by Iruela-Arispe has previously shown that this effect may be related to ADAMTS1 processing of thrombospondins 1&2[6]. It was shown that cleavage of TSP occured between amino acids 311 and 312, similar to our current findings with regards to N-terminal cleavage. Further, TSP cleavage by ADAMTS1 was observed *in vivo* using wound healing models and purified proteolytically released monomers were shown to inhibit endothelial cell proliferation *in vitro*. Thus, it was hypothesized that this may present as a mechanism to release the anti-angiogenic poteintial of matrix bound TSP in the inflammatory setting or serve as an amplification strategy by releasing the individual monomers from the intact TSP trimer.

The ADAMTSs belong to the adamalysin subfamily of the metzincins, or zinc dependent proteases[15]. As such, the current work presented here builds on that previously described, assessing the role of zinc in ADAMTS1 processing of TSP. We show that the addition of physiologic concentrations of zinc[16] lead to additional cleavage of TSP by ADAMTS1. Zinc addition results in cleavage in the C-terminal domain of TSP, with inhibition upon chelation. Therefore these results appear to be specific to zinc. This novel processing of TSP highlights the importance of zinc in ADAMTS1 function and presents a mechanism by which the interaction of the C-terminal domain of TSP with membrane receptor CD47 may be regulated. Additional, more stringent sequencing is needed to specifically identify the C-terminal cleavage site.

The vascular effects of TSP have previously been shown to involve CD36 and CD47[14,17]. TSP inhibition of nitric oxide mediated blood vessel dilation requires CD47

interaction with its C-terminal domain. Alternatively, CD36-TSP interaction has been shown to inhibit growth bFGF and VEGF induced angiogenesis. This interaction is localized to the TSRs of TSP. Thus, zinc mediated C-terminal cleavage of TSP by ADAMTS1 presents a mechanism by which CD47-TSP interaction may be inhibited allowing for CD36-TSP signaling to predominate, thus shifting focus from vascular tone to angiogenesis. It is also possible that C-terminal cleavage of matrix bound TSP is required for release from the cell surface, with subsequent N-terminal cleavage allowing for a 2 stage amplification of cell bound anti-angiogenic stores. Additional study is required to address these hypotheses.

Alternatively, we hypothesize C-terminal cleavage of TSP by ADAMTS1 may occur at sites of inflammation, where matrix metalloprotease levels are known to be elevated. C terminal cleavage may reduce inhibition of vessel dilation by CD47-TSP interaction. This may present a mechanism by which the inflammatory setting may be modulated, allowing for increased edema and immune cell extravasation from vessels. Further work is required to address this hypothesis.

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CHAPTER IV

DISCUSSION

The studies presented in this manuscript examine the importance of the angiogenic switch in the progression of cancer and more specifically the role of TSR-CLESH interaction and processing of TSP by ADAMTS1. The following section highlights the importance of the findings presented and discusses avenues of research which warrant additional inquiry.

The field of anti-angiogenesis has expanded from concept to patient treatment in the forty years since Judah Folkman was told "anti-angiogenic molecules existed only in his mind". Numerous angiogenic inhibitors have been identified and are now under clinical investigation for the treatment of cancer. These include naturally occurring compounds, such as those found in green tea and occurring naturally in the body as well as synthetically manufactured molecules. Additionally, drugs currently on the market have been rediscovered as anti-angiogenic therapies.

As discussed previously in this manuscript, the anti-angiogenic effects of TSP have been localized to the TSRs. Based on these studies three mimetic peptides from this region have been designed and are currently under investigation for the treatment of cancer; ABT-510, ABT-898 and ABT-526[1,2]. ABT-526 is the original TSR mimetic

peptide, off of which ABT-510 and ABT-898 were designed. These compounds have shown increased solubility, potency and slowed clearance compared with ABT-526 (ABT-898 > ABT-510 > ABT-526). Of these, ABT-510 has progressed through phase II clinical trials, showing limited efficacy[3]. Unfortunately, tolerance and conflicting reports with regards to efficacy of these compounds in cancer treatment have been obtained. As such, better characterization of the mechanisms underlying their action is required.

Interaction of the TSRs of TSR containing proteins with the CLESH domain of membrane receptor CD36 initiates an anti-angiogenic cascade resulting in the apoptosis of vascular endothelial cells thereby inhibiting angiogenesis. In previous studies, TSR binding to the CLESH domain of CD36 has been shown to inhibit angiogenesis[4,5,6,7]. We for the first time show direct regulation of tumor angiogenesis and growth by the TSR-CD36 pathway. Past studies have also shown the ability of HRGP, a soluble CLESH homology domain containing protein, to sequester TSR containing proteins, preventing the initiation of the anti-angiogenic CD36 signaling cascade. We verify this effect in the tumor microenvironment showing direct regulation of tumor growth and angiogenesis.

The CLESH domain may therefore serve as an important therapeutic target in the inhibition of cancer angiogenesis. Additional studies utilizing soluble CLESH peptides as well as CLESH binding antibodies and small molecule activators in the regulation of angiogenesis are required. Activation of CD36 signaling or inhibition of HRGP-TSR binding by these compounds may allow for potent inhibition of angiogenesis.

As discussed earlier, ADAMTS1, a TSR containing, protein has been shown to process TSP, releasing anti-angiogenic monomers from the intact trimer. Work by Dr. Iruela-Arispe further showed processing *in vivo* during wound healing, with ADAMTS1

null mice displaying increased vessel density[8]. Work presented here built on these studies, showing additional C-terminal processing of TSP by ADAMTS1 with the addition of zinc to digestion reactions. Further, we hypothesize that C-terminal processing may be required for the release of cell matrix bound TSP.

Thus, it is possible that ADMATS1 processing of TSP allows for a 2 step amplification of its anti-angiogenic potential; first releasing the TSP trimer from the extracellular matrix and second amplifying its inhibitory potential by cleaving the trimer into monomers. This proposed mechanism for increased anti-angiogenic potential by TSP may allow for the modulation of cancer angiogenesis. Treatment with exogenous ADAMTS1 may mediate increased TSR release from endogenous TSP, allowing for increased anti-angiogenic potential. Further investigation is required to evaluate the potential of this strategy.

The studies presented in this manuscript meet the need to better characterize the processes promoting the anti-angiogenic effects of TSR containing molecules, of which 41 have been identified in humans[9]. We should however not overlook the "flip-side" of the coin, instances in which induction of angiogenesis may be beneficial. One of the early studies utilizing pro-angiogenic therapy was in the treatment of coronary heart disease[10]. Following coronary artery bypass surgery patients were locally injected with bFGF. Those injected with bFGF showed increased neovascularization of grafted tissue. Pro-angiogenic therapy has now been employed in the treatment of several pathologic conditions including wound healing, atherosclerosis and cardiovascular ischemia. Thus, inhibition of TSR release and ligation with CD36 may allow for the development of novel pro-angiogenic induction. To this end, exogenous CLESH domain or intact HRGP

administered systemically may allow for the promotion of angiogenesis. Additional study is needed to explore this exciting new possibility.

In conclusion, TSR mediated anti-angiogenesis holds great promise in the angiogenic treatment of cancer. Further insight into the relationship between this domain and the CLESH domain of interacting partners such as CD36 and HRGP will allow us to increase the effectiveness of TSR focused compounds. Additionally, a better understanding of the physiologic processing of intact TSP may provide the opportunity to increase or inhibi the anti-angiogenic capacity of endogenous stores of TSP. Thus, the field of angiogenic inhibition by the TSRs of TSP still has many new and intriguing insights to provide.

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