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Endogenous regulators of angiogenesis – emphasis on proteins with thrombospondin – type I motifs

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Abstract

Angiogenesis has been acknowledged as an important requirement for growth and metastasis of tumors. Complete or partial suppression of vascular growth by a number of different strategies has been consistently associated with suppression of tumor expansion and even reduction of tumor burden. Consequently, identification of the molecular pathways of the angiogenic response has been a major focus of interest in academia and industry. The development of tumor-specific anti-angiogenic therapy was also catalyzed by the finding that inhibitors of angiogenesis appeared immune to the development of drug resistance by the tumor cells, a major restrain in current chemotherapy. Although the full identification of players and their cross-talk is still at its infancy, it appears that partial blockade of one of the steps in the angiogenesis cascade, is sufficient to affect capillary morphogenesis. Thus, suppression of specific integrin pathways or vascular endothelial growth factor signaling have been shown effective in the suppression of tumor-mediated angiogenesis and have led to subsequent initiation of clinical trials.

In addition to the generation of antibodies or chemical mimetics to interfere with particular steps during vascular organization, several endogenous (or physiological) molecules have also been identified. The list of endogenous modulators of angiogenesis is growing and can offer additional and important tool for the generation of therapies to restrain tumor vascularization. This review will focus one group of such molecules which include the thrombospondins and metallospondins, two families of proteins linked by the presence of a conserved anti-angiogenic functional domain.

Introduction

The concept of modulators of the angiogenic response to balance the effect of growth factors has yield much support during the last decade and engendered the concept of the 'angiogenic switch' [1]. The angiogenic switch proposes that in normal stasis, the net 'force' of angiogenic stimulators and inhibitors is zero, or in favor to the inhibitors. In a tumor situation, however, the stimulators outbalance the inhibitors generating a pro-angiogenic response. At this point in time, endogenous inhibitors of angiogenesis can be classified under three major categories:

(1) direct modulators of growth factor signaling, such as soluble flt-l, angiopoetin-2, and soluble neuropilin-1 which function as dominant negative effectors by direct competition of ligand–receptor interactions [2–5];
(2) bioactive cleaved forms of proteins, angiostatin, endostatin, and a 16 kDa fragment of prolactin would be included under this category, these proteins have been mostly found under pathological circumstances [6–9]; and
(3) full length proteins that display angio-inhibitory effects during physiological and pathological conditions. These include platelet factor-4, thrombospondin-1 (TSP1), TSP2 and the metallospondins METH-1 (ADAMTS-1) and METH-2 (ADAMTS-8) [10–13].

The acknowledgement that TSP1 can function as an endogenous inhibitor of angiogenesis is now over 10 years old. Since then, extensive research has been done

to ascertain the mechanism of action that results in such inhibition as well as the identification of functional regions of the molecule that can be target of chemical mimetics for treatment of cancer.

Thrombospondins

Thrombospondins are modular glycoproteins that are composed of multimeric organizations of monomers held together by disulfide bonds. The TSP family consists of of five known polypeptides termed thrombospondins 1–5 [14]. TSP1 and TSP2 are trimeric proteins that are similar in structure whereas TSP3–5 exist as pentamers and differ from TSP1 and 2 in key structural domains that have functional consequences with respect to angiogenesis. In addition to their role in angiogenesis, TSP1 and 2 have been implicated in a number of diverse biological processes including embryonic development, neurite outgrowth/axonal guidance, coagulation and inflammation [15]. In fact, TSP is secreted by a multitude of different cell types including platelets (from which TSP was originally purified), megakaryocytes, chondrocytes, osteocytes, epithelial, endothelial and stromal cells. Despite its diversity in biologic processes, as well as expression in various cell types, much of what is known regarding the biology of thrombospondins particularly TSP1 relates to its role in angiogenesis.

Unique to TSP1 and 2 are the pro-collagen domain and three type 1 repeats which account for the anti-angiogenic properties of these molecules. Peptides from the procollagen and type 1 repeats had been shown to not only inhibit endothelial migration *in vitro* but also inhibit angiogenesis in the corneal pocket assay [16]. Furthermore synthetic peptides of the second type 1 repeats exhibited an antitumor effect that presumably was mediated by an anti-angiogenic mechanism [17]. We have recently conducted a detailed stucture–function analysis of TSP confirming the anti-angiogenic properties previously attributed to the type 1 repeats, but more specifically, identifying two subdomains within the type 1 repeats that independently inhibit angiogenesis [18].

Specifically the 2nd and 3rd repeats have a tryptophan rich region as well as a CD36 binding region that were both capable of inhibiting angiogenesis in the CAM assay to a more significant degree than the intact TSP molecule. Interestingly, mutant peptides incapable of activating the latent TGF-β1 activating sequence were able to inhibit growth-factor induced CAM angiogenesis. Lastly, the tryptophan rich subdomain's angio-inhibitory activity was limited specifically to FGF-2 mediated neovascularization whereas the activity of the CD36 binding domain was observed equally in both FGF-2 and VEGF mediated neovascularization. This distinction has been attributed to the fact that the tryptophan domain has previously been shown to prevent FGF-2 binding to endothelial cells [19,20]. These studies provide evidence to indicate that the antiangiogenic mechanism of action of TSP may be growth factor specific.

Mechanisms of action

Investigations into the mechanism of action of TSP1 have implicted CD36 as the cell surface receptor that mediates its effects on endothelial cells [21]. CD36 is known as a class B scavenger receptor and a collagen binding molecule which had previously been demonstrated to bind to the CSVTCG motif of the second and third type 1 repeats of TSP1 by affinity chromatography [22]. The biologic consequence of this interaction was unknown until experiments demonstrating the inhibitory effect of TSP1 on endothelial cells could be blocked using anti-CD36 antibodies. Moreover, this observed effect could be simulated by using an alternate ligand of CD36 rather than TSP1 to inhibit migration. These results were supported by further experiments using CD36 null mice in which TSP1 was *not* able to inhibit corneal neovascularization [23]. Interestingly, these authors provided evidence to support that a secondary binding motif within TSP1, GVQXR, binds CD36 and mediates the migratory inhibition of TSP1 [21]. These results argue that the anti-angiogenic effects of TSP are receptor mediated rather than by sequestering pro-angiogenic factors or competing with angiogenic growth factors (i.e. FGF-2) for proteoglycans on the surface of endothelial cells. In particular, FGF-2 induced corneal neovascularization was inhibited with activators of CD36 in the *absence* of TSP1 [23]. More recently investigations into the intracellular molecular interactions involving CD36 have determined that TSP1 induces an apoptotic mechanism that involves CD36, Src-family tyrosine kinase $pp59^{fyn}$, group II caspases and p38MAPK [23]. A link between CD36 recruitment and activation of $pp59^{fyn}$ and caspase induction of apoptosis has yet to be determined.

In addition to a CD36 mediated induction of apoptosis, TSP1 displays a number of protein–protein interactions that may or may not have anti-angiogenic consequences. In particular, TSP1 has been found to activate TGF-β1 by releasing it from its latency associated protein, LAP *in vitro* as well as *in vivo* [24,25]. TGF-β1 has been shown to inhibit tumor growth by a yet unknown mechanism; however, evidence exists to suggest that it may be acting via an inhibitory angiogenic mechanism [26]. Alternatively, activation of integrins particularly $\alpha_v\beta_3$ which has been shown to inhibit tumor growth by inducing apoptosis of angiogenic blood vessels may be another mechanism of action as TSP1 has been shown to interact with integrins in the calcium binding type 3 repeats [27]. However, evidence exists to refute the latter hypothesis as peptides lacking the TSP1 integrin binding motifs were able to induce apoptosis of endothelial cells and inhibit angiogenesis [23].

TSP function: Genetic approaches

Genetic approaches to assess the functions of TSP1 and TSP2 have been performed by targeted gene disruption in mice. Because TSP1 has been implicated in a wide array of biologic processes, it was postulated that mice homozygous for a TSP1 deletion would be inviable; however this was not the case. Not only was the TSP1 null mouse viable, but the phenotype did not demonstrate major vascular abnormalities [28]. The most significant defects observed were in pulmonary homeostasis, as these mice developed acute and chronic inflammatory cell infiltrates of unknown etiology. These observations have substantiated previous work implicating TSP1's involvement in modulating the inflammatory/immune response; however these observations were specific to the lung and not found in other organs. In contrast, the TSP2$-/-$ mouse did reveal a vascular phenotype as these mice demonstrated a significant increase in the number of small and medium sized vessels detected by VWF immunostaining in the dermis, adipose and thymus tissues [29]. In addition to this observation the TSP2 null mice displayed a variety of other abnormalities that would not be predicted by previous studies of TSP2 function. These observations include defects in collagen fibrillogenesis and fibroblast attachment, an increase in bone density and an abnormal bleeding time.

TSP in tumor biology

Studies using human epithelial tumor cell lines (lung, breast, bladder) have implicated an anti-tumor effect of TSP1 [30,31]. More definitive *in vivo* studies evaluating the effects of both TSP1 and 2 on tumor growth have shown that these molecules act as potent inhibitors of tumor growth. In correlation with much of the data supporting the inhibitory role of TSP1 and 2 in angiogenesis, their respective anti-tumor effects appear to be mediated by an anti-angiogenic mechanims. Using an orthotopic mouse model of breast cancer, TSP1 was overexpressed by stable transfection in a human breast carcinoma cell line that readily metastasizes both locally and distantly. The study demonstrated a significant inhibition of tumor growth as well as a decrease in number of pulmonary metastases. Immunostaining of mouse endothelial cell markers revealed a significant decrease in vessel density implicating an anti-angiogenic mechanism as responsible for the observed anti-tumor effect [32].

Similarly, Detmar et al. have overexpressed TSP1 and 2 in human squamous cell skin carcinoma cell lines and evaluated their respective effects on tumor growth and angiogenesis using an orthotopic mouse model of skin cancer. Their results not only demonstrated a significant reduction in tumor growth for both TSP1 and 2, but interestingly the anti-tumor effect of TSP2 was greater than TSP1 [33]. Moreover, when overexpressed in combination the two molecules acted synergistically to inhibit tumor growth. Further experiments were performed by these authors to demonstrate that the anti-tumor effect was not the result of an anti-proliferative or apoptosis inducing effect of TSP1 and 2 on the tumor cells, but rather by an anti-angiogenic mechanism. The angiogenic phenotype of these TSP1 and 2 overexpressing tumors was characterized using immunostaining of endothelial cell markers and expressed as vessel density, vessel size and percentage of tissue area covered by vessels. Both TSP1 and 2 induced a decrease in vessel density, average vessel size and vessel area [33,34].

Recently the role of TSP1 as a tumor suppressor in tumor biology has been described. It had been previously shown that the progression of an immortalized, nontumorigenic human skin keratinocyte cell line to a malignant phenotype correlated with the loss of chromosome 15. This phenotype could not only be reversed by transfection of a WT copy of chromosome 15 but that the gene responsible for this reversion

was TSP1. Transfection of TSP1 cDNA into this cell line mimicked the transfer of a WT copy of chromosome 15 and this effect could be reversed by treating the animals with antisense oligonucleotides to TSP1. Further analysis of the tumors and tumor vasculature also demonstrated that the observed anti-tumor effect occurred through an inhibition of angiogenesis rather than a direct effect on the tumor cells [35].

We have more recently generated bigenic animals crossing the MMTV–cneu-transgenic animals with the TSP−/− or TSP overexpressing animals. An impressive reduction in tumor size, number and time of incidence was seen in the TSP1 overexpressing animals in sharp contrast with the animals that lack TSP1 (Rodriguez-Manzaneque et al., submitted for publication). These results together with the large number of xenograft asssays strongly argue that TSP1 and 2 function to restrain vascular growth in tumors.

Metallospondins/ADAMTS

The metallospondin/ADAMTS family of proteins, particularly METH-1/ADAMTS-1 and METH-2/ADAMTS-8 have recently been included in the growing number of molecules found to have anti-angiogenic activities. The cloning of METH-1 and 2 resulted after the screen of libraries for cDNAs that contained the anti-angiogenic domains (second type 1 repeat) of TSP1 and 2 [13].

This growing family of proteins currently comprises 11 members. It is unclear if all the members display anti-angiogenic properties. Typical ADAMTS members contain a pre, pro, metalloprotease-like, disintegrin-like, and a variable number of thrombospondin type 1 repeat domains. Thus the acronym ADAMTS describes their structure as proteins that contain *A D*isintegrin *A*nd *M*etalloprotease with *T*hrombo*S*pondin like repeats. ADAMTS proteases are believed to be involved in the modification of cell surface and extracellular matrix proteins. They exhibit a wide range of tissue distribution which taken together with their multidomain structure implicates them in a number of important biological processes which include but are not limited to angiogenesis, acute inflammation, ovulation, and cartilage remodeling [13,35–39].

ADAMTS-1, the murine form of METH-1 was the first member of the family described. ADAMTS-2/procollagen I N-proteinase (pNPI) is a collagenase responsible for cleaving the pro-form of fibrillar collagens to their monomeric forms prior to polymerization [40]. It is speculated that this enzyme may play a role in other biological processes as its expression has been found to be disproportionately high in relation to the abundance of its substrate in some tissues [40]. The ADAMTS-3 cDNA as been deposited in the gene databank and appears to have a high sequence homology (52%) to pNPI, however no studies exist reporting any functional data [41]. ADAMTS-4/Aggrecanase-I was cloned from IL- 1 stimulated bovine nasal cartilage as a secreted enzyme that cleaves aggrecan, an extracellular matrix component of cartilage whose degradation has been linked to arthritic processes [42]. ADAMTS-5/Aggrecanase II was also cloned from IL-1 stimulated bovine nasal cartilage and has been reported to be active in both a 64 and 50 kDa forms [42]. ADAMTS 6 and 7 cDNAs have been deduced from the gene database. Information describing their relative tissue distributions and mouse/human genome mapping exist; however no experiments have been reported relating functional information about these proteins [43].

ADAMTS-1, mouse ortholog of METH-1

ADAMTS-1 was cloned from a differential display analysis as a gene highly expressed in tumors derived from a cachexigenic clone of murine colon carcinoma cells [36]. Analysis of its expression pattern by Northern blot detected expression in both heart and kidney tissues [36]. Aside from containing three thrombospondin type I motifs at the carboxy terminal end, ADAMTS-1 is distinguished from other typical ADAM proteins in that it contains a secretory signal sequence at the amino terminal end, N-linked glycosylation sites at the carboxy terminal end, and lacks both an epidermal growth factor-like and transmembrane domains thus making it a secreted glycoprotein [36]. Analysis of carboxy terminal deletion mutants have revealed this region to be important for anchoring the secreted protein to the extracellular matrix [36].

The function of ADAMTS-1 is not fully understood; however, evidence exists implicating its role in acute inflammation as (1) it was cloned from a mouse tumor model of cachexia, a pathologic process believed to be mediated by inflammatory cytokines, (2) its expression is upregulated by both IL-1 and LPS, and (3) its transcript has been detected at high levels in human arthritic tissues [36]. Other reports indicate that it is

involved in the ovulation process and its ovarian expression is regulated by progesterone [38,39]. Recently, the knockout of the ADAMTS-1 gene product was performed in the mouse and the analysis of the phenotype reported. ADAMTS-1−/− mice displayed a number of anatomical defects including post natal growth retardation, renal caliceal enlargement and cortico-medullary thining consistent with obstructive uropathy, as well as histologic abnormalities of the uterus and ovaries with concomitant impaired fertility [37]. The significance of these findings relative to the function of ADAMTS-1 as a putative proteolytic modifier of proteins at the cell surface and extracellular matrix is not fully understood

The human ortholog of ADAMTS-1, named METH-1, was cloned in a search for endogenous angiogenic inhibitors that contained the thrombospondin type 1 motifs previously found to exhibit anti-angiogenic properties [13]. A cDNA library of expression sequence tags was screened for genes with homologous sequences to the second type 1 repeats of TSP1 and produced two genes whose amino acid sequences revealed a similar modular structure to other ADAMTS [13]. The names METH-1 and 2 were derived from this structure as molecules that contain *ME*talloprotease and *TH*rombospondin-like repeats. As members of the ADAMTS family, they have been termed ADAMTS-1 and ADAMTS-8, respectively.

Similar to other ADAMTS proteins, METH-1/ADAMTS-1 and METH-2/ADAMTS-8 differ by their number of TSP type I repeats. METH-1 and 2 contains a consensus furin cleavage site located between the prodomain and the catalytic domain.

We have recently demonstrated that METH-1 is secreted as a 110 kDa proform and may undergo processing by furin to its 87 kDa active form extracellularly (Rodriguez-Manzaneque, submitted). In addition, a second processing event occurs at the carboxy terminal end in the spacer region between the first and second TSP type 1 repeats creating a 65 kDa form that is released from the extracellular matrix into the media. This second cleavage event appears to be mediated by matrix metalloproteases as it can be inhibited by MMP inhibitors but not other protease inhibitors.

An analysis of the expression patterns of METH-1 and 2 reveal that they are expressed in a greater number of human tissues than was found with ADAMTS-1 in the mouse; however their patterns are distinct and partially nonoverlapping [13]. METH-1 was detected in most abundance in the adrenal medulla and cortex, heart, kidney, thyroid, liver, placenta and smooth muscle while METH-2 was detected in greatest abundance in the lung, brain and placenta.

In vitro analyses of these proteins have revealed potent anti-angiogenic properties. METH-1 and 2 were found to significantly inhibit endothelial proliferation in a dose-dependent manner to a greater degree than TSP1. This anti-proliferative property was found to be specific to endothelial cells as METH-1 and 2 had no effect on smooth muscle or fibroblast proliferation. Furthermore, both the 87 and 65 kDa processed forms of METH-1 have been recently determined to exhibit similar anti-proliferative activities on endothelial cells (Rodriguez-Manzaneque, submitted). Analysis of these proteins in angiogenic bioassays further established their role as inhibitors of angiogenesis. METH-1 and 2 were both found to significantly inhibit growth factor induced neovascularization in both the CAM and rodent corneal pocket assays to a greater degree than other known inhibitors TSP1 and endostatin [13].

In vivo preliminary data exist to support the role of METH-1 as an inhibitor of tumor angiogenesis as its overexpression in a human breast carcinoma cell line has been found to inhibit tumor growth when implanted subcutaneously in nude mice (D. Carpizo and Iruela-Arispe, unpublished). This anti-tumor effect appears to be mediated by an antiangiogenic mechanism. Figure 1A displays the expression of the METH-1 cDNA in stable transfectants of human T47D breast carcinoma cells. Figure 1B displays their relative effect on tumor growth compared to tumors derived from cells transfected with an empty vector and grown as dual tumors in the same mouse.

Currently it is unclear as to whether the apparent anti-angiogenic effect mediated by METH-1/ADAMTS-1 is secondary to the activity of a specific domain such as the catalytic domain, or the result of a more complex interaction involving the disintegrin-like and/or TSP type 1 repeat domains. Further studies are underway investigating the mechanism of action of METH-1/ADAMTS-1, in particular the substrate of the catalytic domain, as well as protein–protein interactions.

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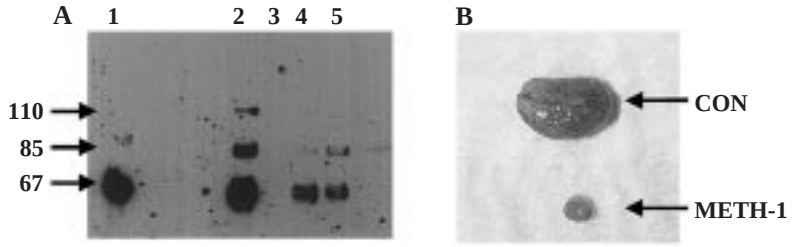

Figure 1. Overexpression of METH-1 restrains tumor growth in xenograph assays. (A) Human cell line T47D was transfected with either vector or a construct containing the coding region of METH-1. Positive clones were isolated and expression of the protein was evaluated by Western blot analysis. Lanes: 1 positive control, 2–5 individual clones. (B) Clone 2 was expanded and cells were injected subcutaneously in nude mice. Controls included cells transfected with vector alone. One pair of such resulting tumors is shown. Note significant difference in tumor growth in the METH-1 transfected cells.

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