Antiangiogenic Domains Shared by Thrombospondins and Metallospondins, a New Family of Angiogenic Inhibitors

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ABSTRACT: The growth of solid tumors has been shown to depend on neovascularization. By understanding the mechanisms that control the neovascular response, it may be possible to design therapeutic strategies to selectively prevent or halt pathologic vascular growth and restrain cancer progression. Thrombospondin-1 is an extracellular matrix protein that among several functions suppresses capillary growth in angiogenesis assays. We have demonstrated that within the context of the mammary gland TSP1 can modulate normal development of blood vessels. Expression of TSP1 in transgenic animals under the control of the MMTV promoter was associated with a 50–72% reduction in capillary growth. In addition, TSP1 reduced tumor size in transgenic overexpressors. The data suggest an important role for TSP1 in modulating vascular growth in both normal and pathologic tissues. The antiangiogenic region of TSP1 has been mapped to the type I (properdin) repeats. To identify novel proteins with such a domain, we have cloned two cDNAs (METH-1 and METH-2) which also have antiangiogenic properties. In addition to carboxyterminal thrombospondin-like domains they also contain metalloproteinase and disintegrin sequences. Expression of both proteins is broad but nonoverlapping. Recombinant fragments from these sequences have strong antiangiogenic potential in the CAM and cornea pocket assays. At the same molar ratio, METH-1 and METH-2 are about 20-fold more potent than TSP1. We predict that these proteins are likely endogenous modulators of vascular growth with relevant therapeutic potential in cancer and other disease states.

INTRODUCTION

Reduction or suppression of tumorigenicity can be accomplished at multiple levels: by direct cell cycle regulation, targeted cellular ablation, control of signal transduction, and/or inhibition of angiogenesis.[1–6] Several investigators have implicated tumor suppressor genes in cell cycle regulation or signal transduction pathways, and considerable effort is being made to identify the critical points in cell transformation that might be sensitive to pharmacologic control. A parallel line of investigation has focused on understanding the regulation of vascular growth. It is recognized that an

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increase in the vascular supply plays a central role in tumor progression and metastasis.[5,7–9] In most tumors, angiogenesis has been acknowledged as a significant indicator of tumor progression that is independent of axillary lymph node status.[9–11] Angiogenesis therapy has recently gained recognized value as a means to suppress tumor growth and metastasis.[6] Several inhibitors and antagonists of VEGF function are currently being tested in clinical trials, and initial evaluations have instigated high levels of enthusiasm (Keystone Symposia, Steamboat, 1998). In this chapter, we provide a general background on angiogenesis inhibitors and introduce a new family of proteins, named metallospondins, that display significant angioinhibitory properties.

ANGIOGENESIS AND THE GROWTH OF TUMORS

The growth of solid tumors strictly depends on new blood vessel formation (reviewed in ref. 5). This relationship between tumor growth and angiogenesis was initially proposed by Folkman and colleagues[12] three decades ago. Thirty years later, their initial observations have gained solid acceptance, and the vascular-density–tumor size relationship has been validated in a variety of tumors including breast carcinoma,[11] melanoma,[13] and brain tumors[14] among others. In fact, the degree of tumor angiogenesis has been identified as a significant and independent prognostic indicator and a requirement for the expansion and metastatic progression of malignant disease.[9,10]

The possibility that tumor progression could be regulated by pharmacologic and/or genetic suppression of blood vessel growth has engendered long-standing interest in the identification of molecules or synthetic compounds that block angiogenesis. The search for angiogenic inhibitors has followed three independent paths:

(1) *identification of inhibitors in tissues that lack blood vessels*: Proteins present in cartilage that antagonize angiogenesis[15–18];

(2) *identification of synthetic or natural substances that normally antagonize the effect of stimulators*: Prostaglandin synthetase inhibitors,[19] protamine (antagonist of heparin),[20] fumagillin (AGM1470), a compound that blocks EC migration and proliferation,[21] antibodies that block VEGF/VPF,[22] and antibodies against $\alpha v\beta 3$;[23]

(3) *characterization of inhibitors that are synthesized or processed as such by tumors*: This approach led to the characterization of angiostatin[24] and endostatin.[25]

At this time, the contribution of angiogenesis to the progression of tumors is widely accepted. The search for additional effective inhibitors is underway in both academic and private sectors. Nonetheless, to date, very few inhibitors have been submitted to clinical trials. Some of the currently recognized angiogenic suppressors are poor candidates for systemic treatment because of their collateral effects. Finally, it is conceivable that specific inhibitors of vessel growth could be efficient in certain pathologies, but ineffective in the control of cancer-mediated angiogenesis. Therefore, it is necessary to identify nontoxic, endothelial-specific, and vascular bed-specific effector molecules that can block rampant growth of tumor-induced capillaries, yet ideally not compromise repair-mediated angiogenesis.

It is our premise that potent and tissue-specific inhibitors are those normally existent *in vivo*. Based on the work of many laboratories, including our own, it has be-

come clear that TSP1 has considerable angiostatic/antiangiogenic activity[26–29] and that, in fact, this molecule is an endogenous modulator of physiologic angiogenesis in the human endometrium and mammary gland.[29] For almost 10 years, our laboratory and several others have focused our research on the ability of TSP1 and peptide mimetics to regulate angiogenesis *in vivo*[30] and to suppress the growth of tumors. Experiments using xenograph assays as well as several *in vivo* models of angiogenesis have consistently supported TSP1 as a negative modulator of angiogenesis with the ability to suppress tumor growth.[31] Together, these findings argue strongly for studies on TSP1 and TSP1-related gene products that could be effective in controlling vascularization in specific settings.

The region responsible for the antiangiogenic properties of TSP1 has been confined to the second and third type 1.[30,32] In an effort to identify new inhibitors of angiogenesis that might contain the TSP1 antiangiogenic repeats, we have screened a large database of ESTs and identified five previously uncharacterized cDNAs. Full-length sequence revealed that two of those cDNAs are highly similar (52% at the amino acid level) and, in addition to the TSP repeats, contain metalloproteinase and disintegrin motifs.[33] On the basis of their structure, we named them METH-1 and METH-2 (ME for metalloproteinase and TH for thrombospondin). Interestingly, another protein with the same structural features was identified and named pNPI for pro-collagen N propeptidase.[34] All three proteins have a metalloproteinase and disintegrin domain followed by a varied number of TSP/type 1 repeats. The metalloproteinase-disintegrin motif is reminiscent of the ADAM family of growth regulatory genes. Recently, the murine homolog of METH-1 was published and named ADAMTS.[35] A comparison between murine and human genes showed 84% similarity at the amino acid level.[33] We consider all of these proteins as members of a new family of proteins which we have named metallospondins. We believe that this family has at least four additional members, given the ETS Genebank database. The general structure of metallospondins is presented in FIGURE 1.

The TSP repeats in METH-1 and METH-2 are more similar to the second and third TSP repeats of TSP2, the region known to display antiangiogenic function. A comparison of the sequences is provided in FIGURE 2.

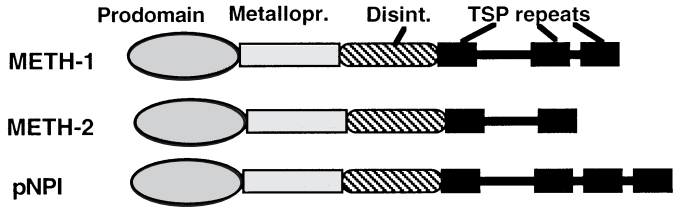

FIGURE 1. Protein structure of metallospondins. The basic structure of METH-1, METH-2, and pNPI includes a prodomain followed by a metalloproteinase domain with a typical zinc-binding motif, disintegrin (cysteine-rich) region, and a variable number of TSP/type 1 domains, 3 in METH-1, 2 in METH-2, and 4 in pNPI.

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   *  *  *  *              *                          *    *
SPRWSLWSTWAPCSVTCSEGSQLRYRRCVGWNGQCSGKVA-PGTLEWQLQACEDQPCCP  PROPERDIN
DGGWSHWSPWSSCSVTCGDGVITRIRLCNSPSPQMNGKPC--EGEARETKACKKDACPI  TSP-1
DGGWSHWSPWSSCSVTCGVGNITRIRLCNSPVPQMGGKNC--KGSGRETKACQGAPCPI  TSP-2
HGSWGMWGPWGDCSTRCGGGVQYTMRECDNPVPKNGGKYC--EGKRVRYRSCNLEDCP   METH-1
DGGWAPWGPWGECSRTCGGGVQFSHRECKDPEPQNGGRYC--LGRRAKYQSCHTEECP   METH-2
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FIGURE 2. Type 1/TSP repeats in several proteins. Sequence (single amino acid code) comparison of TSP/type 1 repeats in properdin (first repeat), TSP1 (second repeat), TSP2 (second repeat), METH-1 (first repeat), and METH-2 (first repeat). Common tryptophane (W) and cysteine (C) residues are identified by an *asterisk*.

TSP OR TYPE I REPEATS AND ANGIOGENESIS

The type I repeats of TSP1 have been characterized extensively.[36–38] The sequences have been shown to (1) bind heparin in three regions (KRFK, WSPW, and CSVTCG), (2) activate latent forms of TGF-β via a nonproteolytic pathway by the cooperation of two sequences (K/R-F-K/R and WSPW), (3) bind sulfatide, (4) promote cell adhesion, and (5) inhibit angiogenesis through the CSVTCG sequence. The sequences of CSVTCG and WSPW in METH-1 may well have similar biological functions.

Interestingly, the TSP or type 1 motif is present in several molecules, including TSP-2, complement components C6 to C9,[38] F-spondin,[39] UNC-5 in *Caenorrhabditis elegans,*[40] proteins related to connective tissue growth factor (including CEF-10, CY-61, FISP-12, and NOV),[41–46] and in various parasite proteins such as circumsporozoite and thrombospondin–related anonymous protein (TRAP) in *Plasmodium.*[47–48] The function of the TSP repeats in most of these proteins has not been studied. Nonetheless, removal of the TSP region from the circumsporozoite protein in *P. falciparum* has been shown to impair binding to its receptor on live cells.[49] In properdin, this repeat binds to sulfated glycoconjugates[50–54] and to C3b.[55]

Are all proteins with TSP repeats antiangiogenic? With the exception of studies with TSP1, this question has not been addressed. Because of the identification of METH-1 and METH-2, we have partially addressed the issue by generating recombinant fusion proteins containing TSP repeat sequences from TSP1, properdin, METH-1, and METH-2. FIGURE 3 shows the degree of purity of the recombinant proteins used in angiogenesis assays. All proteins were evaluated for angiogenic activity side-by-side and at the same molar ratio.

Fusion proteins as well as GST alone (negative control) was used on chorioallantoic membrane assays. In this assay, a pellet containing polymerized collagen and angiogenic growth factors (VEGF and FGF-2) was applied to the surface of the CAM to induce a neovascular response in 24 hours. CAM-derived vessels grow against gravity and invade the acellular collagen gel. In the experimental pellets, recombinant proteins were also incorporated to challenge the ability of growth factors to elicit an angiogenic response. When an inhibitor was present, colonization of the collagen gel by vessels was significantly diminished or absent. The degree of angiogenesis was assessed by injection of high molecular weight FITC-dextran into the chicken embryonic circulation. The intensity of fluorescence (equivalent to vascular density) displayed in a 250-μm^2 area was determined using Image 3 software in the

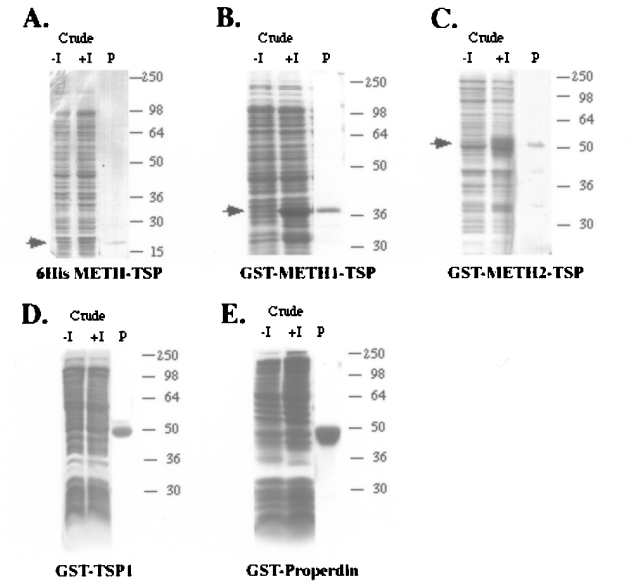

FIGURE 3. Purification of recombinant proteins. SDS-polyacrylamide gel electrophoresis analysis of total cell lysate and purified *Escherichia coli* recombinant proteins stained with Coommassie blue. Molecular weight of standards is indicated on the *right*. *Lane 1*, total cell lysate prior to IPTG induction; *lane 2*, total cell lysate after IPTG induction; *lane 3*, purified protein. (**A**) 6His-METH1, a histidine-tagged protein containing the first TSP-domain of METH-1; (**B**) GST-METH1, a GST fusion protein containing the first TSP-like domain of METH-1; (**C**) GST-METH-2, a GST fusion protein containing the first TSP-like domain of METH-2; (**D**) GST-TSP1, a GST fusion protein containing the second and third TSP-like type 1 domain of TSP1; and (**E**) GST-properdin, a GST fusion protein containing the first and second TSP-like type 1 domains of properdin.

growth factor-containing pellets and was considered a 100% response. The degree of inhibition of angiogenesis was determined by comparing this value to those displayed in the experimental pellets. FIGURE 4 shows the degree of vascular response in a 250-μm^2 area after 24 hours of treatment. A dense network of capillaries was detected in the pellets containing growth factors and GST (FIG. 4A). By contrast, GST alone did not promote an angiogenic response (FIG. 4B). A variable degree of inhibition of angiogenesis was seen in the presence of TSP1, METH-1, and METH-2 fusion proteins (FIG. 4C-E). By contrast, the properdin fusion protein did not suppress the neovascular response mediated by the angiogenic stimulators (FIG. 4F). Results were consistent in seven independent experiments. In addition to the experiments performed with the METH-1 and METH-2 type 1 repeats, the entire proteins also displayed angioinhibitory activity.[33]

These results would argue that the presence of TSP repeats alone does not render a protein an angiogenic inhibitor. It appears that differences in the primary structure of these proteins, or perhaps adjacent sequences, participate in the overall antiangiogenic function displayed by these proteins.

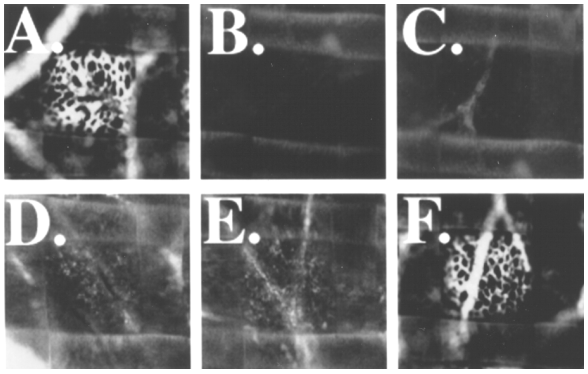

FIGURE 4. Effect of recombinant TSP-repeat fusion proteins on growth factor-mediated angiogenesis. For these experiments 12-day-old white Lenhorn chicken embryos were used. Vitrogen pellets and injection of FITC-dextran was performed as previously described.[30,57] Assays were carried out for 24 hours. Confocal images within the vitrogen pellet are shown in a window of 250 μm^2. Pellets correspond to: (**A**) VEGF (250 ng/pellet) and FGF-2 (50 ng/pellet) in the presence of GST; note the dense network of capillaries; (**B**) GST alone (negative control) shows no capillary growth; all subsequent pellets contain the same mixture of VEGF and FGF-2 as in **A** in addition to: (**C**) METH-1/GST fusion protein (7 μg/pellet); (**D**) METH-2/GST fusion protein (5 μg/pellet); (**E**) GST-TSP1 (5 μg/pellet); and (**F**) GST-properdin (5 μg/pellet).

Although we have shown that metallospondins have angio-inhibitory activity *in vivo*, the efficacy of these proteins in tumors might be variable. We and others have accumulated sufficient data in animal studies to argue that TSP1 constitutes a likely candidate for antiangiogenic therapy in tumors.[30,31] Whether METH-1 and METH-2 will show the same efficacy within the context of a tumor is still speculative. In angiogenesis assays, both METH-1 and METH-2 intact, full-length proteins appear more potent than either TSP1 or endostatin.[33] Experiments using xenograph assays are currently underway and will determine their efficacy in reducing tumor growth.

CONCLUDING REMARKS

The relationship between tumor progression and induction of a neovascular response has been well documented by several investigators in many independent laboratories. Inhibitors of angiogenesis may prove effective as adjuvant therapy in cancer patients, and several animal studies have demonstrated their efficacy in inhibiting tumor growth or inducing tumor regression.[24,25,31] Furthermore, antiangiogenic treatment of tumor-bearing mice does not induce acquired drug resistance, offering a valuable advantage over conventional cytotoxic therapies that target tumor cell growth.[56] Therefore, the identification and study of angiogenesis inhibitors can provide valuable pharmacologic information to prevent neovascularization in tumors. Here we have introduced a new family of inhibitors, metallospondins. Along with these molecules, novel modulators of endothelial cell function and angiogenic response appear frequently in the literature. Future research efforts should be direct-

ed towards further understanding the biology of these inhibitors, their specificity for tumor and other vascular beds, their relative potency, and their mechanism of action.

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