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CD36: A CRITICAL ANTI-ANGIOGENIC RECEPTOR

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TABLE OF CONTENTS

1. ABSTRACT

Thrombospondin-1 (TSP-1) is a potent inhibitor of angiogenesis *in vivo* and of microvascular endothelial cell responses to angiogenic factors *in vitro*. CD36 is the cellular receptor for TSP-1 on microvascular endothelium and is necessary for its anti-angiogenic activity. The anti-angiogenic activity of TSP-1 is contained in a structural domain known as the TSP type I repeat (TSR-1). TSR-1 domains occur in many other proteins, some of which have also been shown to have anti-angiogenic activity. Structure-function analyses have determined that binding of TSP-1 to CD36 is mediated by interaction of the TSR-1 domain of TSP with a conserved domain called CLESH-1 in CD36. Histidine rich glycoprotein, a plasma and cellular protein that blocks the binding of thrombospndin-1 to CD36, inhibits the antiangiogenic response to thrombospondin and may serve to modulate the thrombospondin/CD36 anti-angiogenic pathway. Several ***in vivo*** models support the role of the TSP/CD36 system in angiogenesis and tumor growth and provide evidence that the CD36 antiangiogenic pathway offers attractive therapeutic targets.

2. INTRODUCTION

New blood vessel growth is regulated by a well-orchestrated balance between pro- and anti-angiogenic factors (1). Since endothelial cells in normal adult tissues are quiescent, initiation of angiogenesis requires activation of a process mediated in most forms of post-natal angiogenesis by release of endothelial cell mitogens, such as basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF), from ischemic tissues (2). Pioneering work by Folkman and colleagues has shown that tumors cannot grow beyond 1-2 mm in size in the absence of the ability to promote ingrowth of new blood vessels (3). If the angiogenic process remains unchecked, nutrients and growth factors supplied by new blood vessels will allow tumors to expand and metastasize. Furthermore, tumor masses which may remain dormant and undetectable for years may suddenly acquire the ability to promote neovascularization, a process that has been termed angiogenic "switch" (4). These findings have led to the hypothesis that inhibition of angiogenesis may interfere with the development and progression of cancer. Studies have shown that in murine models, inhibition of angiogenesis may actually lead to regression of large tumor masses, suggesting that angiogenesis is an active, dynamic process in tumors (5). Recent attention has therefore focused on the identification and characterization of natural inhibitors of angiogenesis and their receptors, which provide attractive targets for therapeutic intervention. This review will focus on the role of a specific microvascular

Figure 1. Domain structure of TSP-1. The modular structure of a TSP-1 monomer is shown. Homotrimeric TSP-1 is formed through disulfide linkages at the N-terminus. The anti-angiogenic activity of TSP-1 is localized to the thrombospondin structural repeats (TSR, shown in green), the region to which CD36 binds.

endothelial cell receptor, CD36, as a critical determinant of the anti-angiogenic switch.

3. THROMBOSPONDIN

3.1. The identification of thrombospondin-1 as a natural inhibitor of angiogenesis

Early work on anti-angiogenesis factors identified angiostatic activity in extracts of avascular tissues, steroid compounds, protease inhibitors, anti-inflammatory agents, and heparin fractions (6). Much of the inhibitory activity of these agents is probably related to indirect effects, such as disruption of the extracellular matrix modifications needed for successful angiogenesis. Thrombospondin-1 (TSP-1) was the first protein to be recognized as a natural inhibitor of angiogenesis by a genetic strategy to identify tumor cell-secreted inhibitors of FGF-mediated corneal angiogenesis. Several observations suggest that TSP-1 may function as a physiologic regulator of angiogenesis. TSP-1, a major constituent of platelet alpha-granules, is secreted upon platelet activation and thus present at sites of vascular injury. It is also a highly regulated component of the extracellular matrix secreted by fibroblasts, smooth muscle cells, and endothelial cells in response to vascular injury, growth factors and cytokines. A 140kd fragment of TSP-1 was discovered to be an inhibitor of angiogenesis by Bouck and colleagues using a somatic cell genetic strategy based on demonstrating suppression of the angiogenic phenotype of tumor cells by fusion with normal cells (7). Subsequent studies *in vitro* studies with cultured vascular endothelial cells have shown that TSP-1 inhibits proliferation, migration, and tube formation in response to multiple angiogenic stimuli bFGF and VEGF (8). Mice rendered null for TSP-1 have increased vascularization in a number of tissues, and display prolonged neovascularization in response to wounding, further supporting an important role for TSP in regulation of blood vessel growth and development (9). Targeted overexpression of TSP-1 in mice inhibits wound healing and tumor growth (10).

TSP-1 is a multifunctional 450 kD adhesive glycoprotein that is secreted by activated platelets and is expressed in by a variety of normal vascular cells, including endothelial and smooth muscle cells (11). Its production by vascular cells is highly regulated and is dramatically increased in response to growth factors and cytokines, including FGF. It is thus present in large amounts in wound tissue and in certain tumor beds. It is the first described member of a family of 5 related gene products, now called TSP-1, -2, -3, -4 and COMP (12). TSP-1 interacts with many matrix constituents, including heparan sulfate proteoglycans, fibronectin, and collagen, and probably plays a role in matrix stability and remodeling. Evidence from TSP-1 null and transgenic mice suggests that this is particularly important in bone matrix and during vascular wound matrix remodeling (9, 10). TSP-1 can also interact with at least 12 specific cellular adhesion receptors, including CD36, alpha-v integrins, certain beta-1 integrins, syndecan, and integrin associated protein (IAP; CD47). It can function as an adhesion molecule, promoting tumor cell-matrix interactions and platelet aggregation, but it also has anti-adhesive properties for some cells, leading, for example, to disruption of endothelial cell focal adhesion plaques (13). On macrophages, TSP-1 mediates recognition and phagocytosis of apoptotic leukocytes, thus participating in the later stages of the inflammatory response and limiting pro-inflammatory influences (14). TSP-1 interacts with several proteases thought to be involved in angiogenesis, including plasminogen, urokinase, matrix metalloproteinase, thrombin, cathepsin and elastase (15). It also binds TGF-beta with high affinity and can efficiently convert the latent form into the active form (16). TGF-beta is found in large amounts in injured vessels where it promotes collagen production and matrix deposition by vascular cells, suggesting that TSP activation of TGF-beta is important in vascular injury responses.

3.2. Structure-function properties of thrombospondins

The ability of TSP-1 to effect so many disparate functions relates directly to its complex structure (17). TSP-1 is a homotrimeric protein organized as a series of discrete modules in linear array. Studies utilizing protease digestion, monoclonal antibodies, synthetic peptides, and recombinant TSP-1 fragments have shown that each of these domains is responsible for specific functions (figure 1). The most N-terminal of these modules is a domain that mediates heparin binding and the disulfide bond dependent trimerization of the TSP-1 monomers. Immediately adjacent to the heparin binding domain is a cysteine-rich region with homology to procollagen. Following that are 3 copies of a domain called the type I repeat (TSR-1), sequences of 50-54 amino acids that are homologous to malaria and complement proteins. Next are 3 copies of type II repeats bearing EGF-like homology, and then 7 type III repeats that share homology to calcium binding sites in many other proteins. The last type III repeat contains the integrin binding domain, RGDA. This is followed by a unique globular carboxy terminal domain containing the binding site for integrin-associated protein. The apparently contradictory studies showing that TSP-1 has both pro-adhesive and anti-adhesive properties can be explained by the existence of multiple TSP-1 cellular adhesion receptors, each with specificity for a different TSP-1 domain, and each with its own unique pattern of cellular expression. The TSR-1 repeats are most relevant to angiogenesis and are each encoded by a separate exon, suggesting that each has an independent folded structure.

Initial studies with proteolytic digests of TSP-1 revealed that its anti-angiogenic activity was contained within a 140kD fragment beginning just C-terminal to the heparin binding globular domain, and was maintained in

the 70kD stalk region consisting of the procollagen domain, the three TSR-1 domains, and 2 of the EGF domains. Shorter fragments lacking the EGF domains did not lose activity. Tolsma *et al.* synthesized synthetic peptides based on sequences from the anti-angiogenic regions and determined that short sequences from the second and third TSR-1 domains were potent inhibitors in *in vivo* and *in vitro* assays of angiogenesis (18). These have in common a CSVTCG sequence found in CD36-binding malaria peptides, as well as a distal GVXXR sequence. A peptide from the first TSR-1, containing the sequence CSTSCG, and that also included the distal GVXXR sequence, was inactive in most assays.

The studies of Tolsma *et al.* do not rule out the possibility that TGF-beta activation might contribute to the anti-angiogenic activity of TSP-1. The TGF-beta binding and activation domain also appears to be contained within the type I repeats, but it is distinct from the CD36 binding domain (19). Murphy-Ullrich and colleagues have identified a TSP-1 sequence KRFKQDGGWSHW involved in TGF-beta activation and have shown that the KRFK tetrapeptide sequence is necessary (20). TSP-2, however, lacks this domain and maintains anti-angiogenic activity equivalent to TSP-1 (21). A sequence from the procollagen domain, NGVQYRN, was also shown to be anti-angiogenic in these studies. The mechanism of this latter domain activity is unclear, but again this domain is missing in TSP-2.

Tan *et al.* recently reported the first high resolution structural data for TSP-1, and showed that the TSR is a highly structured domain comprised of three antiparallel strands held together by a series of interlocking stacks of amino acid side chains. These side chains are made up of six alternating layers of tryptophans (W layer) and arginines (R layer) sandwiched between cystine disulfides, forming a cationic face containing a novel groove-like structure. One hypothesis emerging from these studies is that this groove forms a recognition site for ligands such as an anionic disaccharide unit from a right-handed spiraling heparin molecule which could "fit" well into the cationic grove formed in the 20-Å distance between the first W layer and the third R layer (22).

Identification of the TSR-1 domain as the active anti-angiogenic component of TSP-1 raises the question of the role of other TSR-1 containing proteins in regulating angiogenesis. TSP-2, like TSP-1, contains three TSR-1 domains and is anti-angiogenic in corneal pocket and *in vitro* assays. TSP-2, which has a more restricted pattern of expression than TSP-1, is expressed in dermal fibroblasts and has been shown to be upregulated in skin inflammation and skin carcinogenesis. In cutaneous wound healing, TSP1 and TSP2 are expressed at different time points: TSP1 mRNA is highest during the first 24 hours, while TSP-2 appears 3 days after wounding, with maximal expression at day 10. Studies of mice with targeted disruption of TSP-1 or TSP-2 substantiate the distinct roles of these proteins in maintaining normal angiogenic balance: TSP-1 knockouts display prolonged neovascularization in response to wounding and susceptibility to pulmonary infections, while TSP-2 knockouts demonstrate an increased density of blood vessels in many tissues, prolonged bleeding time, and abnormal tensile strength of the skin (23,24).

In experiments designed to identify novel angiogenesis inhibitors, Iruela-Arispe screened a cDNA library with sequences encoding the second and third TSP-1 TSR-1 domains. She identified two novel gene products, METH-1 and METH-2, and showed that they were anti-angiogenic by several assays, including the corneal pocket assay (25,26). METH-2 is highly expressed in the endometrium and is upregulated with ovulation, while METH-1 is more widely expressed. These are now known to be members of the ADAMS-TS gene family (ADAMS-TS-1 and -8), a group of at least 11 genes encoding proteins containing a metalloproteinase domain, a disintegrin domain, and one or more TSR-1 domains.

Angiostatic TSR-1 domain containing proteins have also been described in the brain. Glioblastomas are among the most vascularized tumors, and mutations in p53 appear to play a role in their progression. Nakamura and colleagues hypothesized that p53 target genes that regulate angiogenesis may play a significant role in progression of these neoplasms. Using a p53-tagged site as a probe, they identified a brain-specific target gene which contained TSP-1 type I repeats, and called it BAI1 (brain angiogenesis inhibitor 1) (27). They have since expanded their findings, and determined that BAI1 is one of a family of three transmembrane proteins with multiple TSR-1 domains (28).

4. THE ROLE OF CD36 IN REGULATING ANGIOGENESIS

4.1. CD36: a multifunctional glycoprotein

Although as many as 12 different potential cellular receptors for TSP-1 have been identified, evidence from Bouck and from our laboratory has implicated CD36, an 88 kDa transmembrane glycoprotein, as the critical anti-angiogenesis receptor for TSP-1 (29) . CD36 was first described as a platelet surface protein of unknown function. We now know that CD36 is expressed in a diverse array of cells and tissues, including microvascular endothelium, dendritic cells, monocytes/macrophages, erythroid precursors, and specialized epithelia of the retina and breast (30-32). It is a single chain, heavily glycosylated transmembrane protein of molecular weight 78-88 kDa, depending upon the cell type from which it is isolated. Most of the CD36 protein is oriented extracellularly. CD36 is the defining member of a small gene family which in humans includes lysosomal integral membrane protein (LIMP)-2 and scavenger receptor B1(SR-BI) (33). The primary structure of CD36 is highly conserved across mammalian species and homologs have been described in *Drosophila* and *C. elegans*. CD36 family members have in common the recognition of lipid and modified lipid, that can be expressed in the membranes of apoptotic cells and on lipoproteins. In addition to its function as a receptor for TSP-1, CD36 also functions as a scavenger receptor, mediating recognition and internalization of oxidized lipoproteins, apoptotic cells, and free fatty acids.

Unlike most other TSP receptors, endothelial cell expression of CD36 is restricted to microvascular cells, the cells from which new blood vessels arise (32). In addition, the region on TSP recognized by CD36 is the same domain shown to have anti-angiogenic activity; i.e. the CSVTCG type I repeat (34). Studies with monoclonal antibodies and synthetic peptides have clearly identified the CSVTCG sequence found in the 2nd and 3rd TSR-1 domain as the binding domain on TSP-1 for CD36. These peptides bind to CD36, inhibit TSP-1 binding to purified CD36, and block the interaction of CD36 expressing cells with TSP-1(35). To identify the corresponding binding domains on CD36 for TSP-1, we used a panel of overlapping recombinant glutathione-S-transferase/CD36 fusion peptides that span the entire length of CD36. We found that the oxidized LDL binding domain and TSP-1 binding domain are separate and independent, and that the region from amino acid 93-120 satisfied all the requirements for a TSP-1 binding domain. This peptide bound TSP-1 with affinity similar to intact CD36, inhibited TSP-1 binding to CD36, and blocked TSP-1 binding to cells transfected with the CD36 cDNA. Peptides from this region have been shown to inhibit CD36-dependent TSP-1 functions *in vivo* (36). Leung *et al* have shown that sequences downstream of amino acid 120 may regulate the affinity of TSP-1 for CD36, suggesting that interaction of TSP-1 with CD36 may occur via a two step mechanism (37). In addition, Asch *et al.* reported that extracellular phosphorylation of tyrosine at position 92 (immediately adjacent to the binding domain) inhibits TSP-1 binding and may occur *in vivo* to regulate function (38).

4.2. The anti-angiogenic activity of TSP-1 is mediated by CD36

There is substantial *in vitro* and *in vivo* evidence demonstrating that CD36 is the anti-angiogenic endothelial cell receptor for TSP-1. Blockade of the CD36-TSP interaction with specific monoclonal anti-TSP or anti-CD36 antibodies, or with peptides derived from the region of CD36 that binds TSP blocked the TSP-induced anti-proliferative, anti-migratory, and anti-tube formation response of cultured microvacular endothelial cells exposed to angiogenic stimuli such as bFGF. These reagents had no effect on the anti-angiogenic activity of angiostatin. Furthermore, transfection of CD36 cDNA into large vessel endothelial cells that did not exhibit an anti-angiogenic response to TSP, conferred that ability, and other CD36 ligands, including oxidized LDL and a multivalent IgM monoclonal antibody, were shown to have anti-angiogenic activity (29).

Histologic examination of the brain of CD36 null mice demonstrated an increase in the number of blood vessels, similar to that reported in TSP-1 null mice, evidence that the anti-angiogenic activity of TSP-1 is mediated through CD36 *in vivo*. A corneal angiogenesis model was used to examine directly the role of CD36 in mediating the anti-angiogenic activity of TSP-1. When sucralfate/Hydron implants containing growth factors such as bFGF were implanted into the cornea of a mouse, they induced a vigorous angiogenic response, with growth of new blood vessels from the limbus towards the pellet. When TSP-1 was added to the to the corneal implant along with bFGF, there was significant inhibition of angiogenesis in wild type mice. In contrast, in CD36 null mice, TSP-1 failed to inhibit angiogenesis in response to bFGF. Angiostatin, an inhibitor of angiogenesis which is not known to interact with CD36, retained anti-angiogenic activity in both wild-type and CD36 null mice. The lack of inhibition of angiogenic response to TSP-1 by CD36 null mice is further evidence that the presence of CD36 is necessary for the ant-angiogenic activity of TSP-1 (39).

4.3. Downstream effects of TSP-1 interactions with CD36

TSP-1 inhibits angiogenesis by inducing apoptosis of endothelial cells (EC), and is one of the first natural inhibitors of angiogenesis for which a signaling pathway has been outlined. Recent work has shed light on the pathways by which CD36 by TSP-1 on microvascular EC inhibits a growth factor-mediated angiogenic signal and diverts the cell toward an anti-angiogenic, apoptotic pathway.

CD36 has only a short cytoplasmic domain of 11 amino acids that lacks intrinsic kinase or phosphatase activity. CD36 co-precipitates with, the non-receptor protein tyrosine kinases fyn, lyn, and yes (40). Treatment of cultured primary human microvascular EC with TSP-1 led to apoptosis and was associated with the recruitment of the tyrosine kinase p59fyn to CD36 complexes and increased p59fyn phosphorylation. Two stress-activated kinases, p38 mitogen-activated kinase (MAPK) and c-Jun N-terminal kinase (JNK) have been shown to be critical for apoptosis-mediated angiogenesis inhibition by TSP-1. TSP-1 induced EC apoptosis via a fyn-dependent pathway involving activation of caspase-3-like proteases and p38 MAP kinase (39). These findings were supported by *in vivo* evidence using corneal angiogenesis assays, in which the anti-angiogenic activity of TSP-1, but not angiostatin, was lost in fyn null mice. Similarly, TSP-1-mediated JNK activation by TSP-1 in microvascular EC was blocked by antibody to CD36, and mice null for JNK were resistant to the anti-angiogenic effect of TSP-1 (41).

Regulation of neovascularization depends on the regression of new blood vessels through apoptosis of EC. TSP-1-induced apoptosis of EC has been shown to be dependent on Fas, Fas ligand (FasL), and caspase 8. The binding of FasL to its receptor Fas, a member of the tumor-necrosis factor (TNF) receptor superfamily, leads to activation of caspase-8 and initiation of an apoptotic cascade. Volpert *et al* showed that EC treated with the angiogenic factors VEGF, bFGF and IL-8 displayed more Fas on their surface. Treatment with TSP-1, on the other hand, induced the expression of FasL, leading to induction of apoptosis. These studies suggest that new vessels are "primed" for destruction by a balance of pro-and anti-angiogenic factors. Further evidence to support this model comes from studies of Fas- and FasL-deficient mice, which were shown to have increased microvascular densities in the dermis and retina (42).

The association of CD36 with tyrosine kinases may involve co-localization in caveolae, the cholesterol and sphingolipid-rich membrane microdomains that may

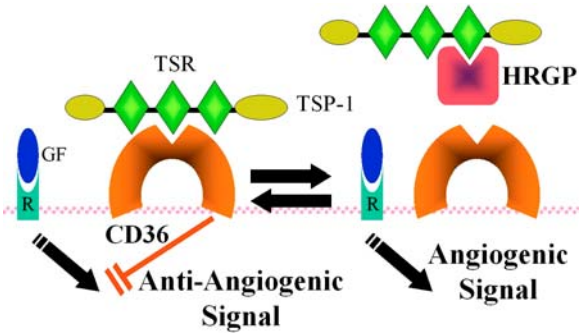

Figure 2. Modulation of angiogenesis by histidine rich glycoprotein. Angiogenesis initiated by the binding of an angiogenic growth factor (GF) to its receptor (R) is inhibited by TSP-1 through the interaction of the TSR (green diamonds) with the CLESH-1 domain of the signaling receptor CD36 (left). HRGP, which also contains the CLESH-1 motif, binds TSP-1, inhibiting the interaction of TSP-1 with CD36 thereby inhibiting the anti-angiogenic effect of TSP-1 (right).

function to localize signaling molecules, such as src-kinases, MAP kinases, and small molecular weight GTPases, with membrane receptors and GPI-anchored membrane proteins. Roles for caveolae in membrane docking and fusion, growth factor signaling, and integrin signaling have been proposed. The major structural components of caveolae are caveolins, small (22-25kD) membrane proteins that span the membrane several times and contain a cytoplasmic "scaffolding" domain that can interact with several intracellular signaling molecules. Lisanti and *et al* have shown that immunoprecipitation of canine kidney epithelial cells with a specific murine anti-caveolin antibody co-precipitated an 88kD protein that on sequence analysis was CD36 (43). CD36 co-precipitated with anti-caveolin-1 in microvascular EC, and incubation of oxidized LDL with microvascular EC resulted in cholesterol depletion from caveolae. The latter effect resulted in loss of localization of endothelial nitric oxide synthase (eNOS) and impaired eNOS activation, but was blocked by anti-CD36 antibodies.

5. THE ROLE OF HISTIDINE-RICH GLYCOPROTEIN AS A REGULATOR OF TSP-CD36 INTERACTIONS

5.1. CD36-like homology motifs in HRGP

The TSP-1 binding domain in CD36 is conserved in CD36 gene family members lysosomal membrane integral protein II (LIMPII) as well as other TSP-binding proteins such as the gp120 envelope glycoprotein of HIV and histidine rich glycoprotein (HRGP). This region, which we have termed the CLESH-1 (CD36, LIMPII, emp Structural Homology) domain, may function in the regulation of TSP-1/CD36 anti-angiogenic interactions (44).

HRGP is a 66 kDa plasma protein with an unusually high histidine and proline content that circulates in relatively high concentrations (1.5μM) and was shown many years ago to bind TSP-1 (45). The structure of HRGP consists of a number of discrete domains including two cystatin-like domains at the amino terminus, and a histidine-proline rich region (46). The carboxy terminus contains the binding site for plasminogen as well as a region with significant homology to the TSP-1 binding site of CD36. Although HRGP has no known protease activity, it is related to the cystatin superfamily, which includes high molecular weight kininogen (HMWK). There are two putative CLESH-1 motifs flanking the histidine/proline –rich region.

HRGP may interfere with fibrinolytic activity by binding to the lysine binding site on fibrinogen and interfering with plasminogen binding to fibrin. HRGP, TSP, and plasminogen form a tri-molecular complex which retains plasmin generation activity in the presence of tissue plasminogen activator (TPA) (47). Surface bound HRGP can accelerate the activation of plasminogen by TPA, suggesting a role for HRGP in the fibrinolytic system (48). Similarly, HRGP binds to fibrinogen and fibrin in a divalent cation-dependent manner, and can form a trimolecular complex with fibrinogen and plasminogen (49). It has also been shown that HRGP effects on fibrin polymerization may lead to prolongation of the thrombin time *in vitro* and may alter the structure of the fibrin clot. Further studies showing binding of HRGP to heparin and other glycosaminoglycans also suggest a role in the modulation of the coagulation/fibrinolytic system.

5.2. HRGP binding to TSP-1

HRGP binds to TSP-1 saturably, reversibly, and with high affinity (7nM) (45). Using binding studies in the presence of peptides derived from the TSR, we found that HRGP binding to TSP-1 was inhibited by the peptide CSVTCG but not by scrambled or mutated peptides. As further evidence for the specificity of this binding activity, we found that binding of HRGP to TSP-1 was not inhibited by peptides derived from the TGF-β-binding sequence (GGWSHW), heparin-binding sequence (SHWSPWSS), the C-terminal domain (RFYVVMWK), or an RGDS peptide derived from the type 3 repeats. Binding of HRGP to TSP-1 was significantly decreased in the presence of anti-CSVTCG antiserum and completely abolished by the CSVTCG peptide (50).

5.3. HRGP inhibits the anti-angiogenic activity of TSP-1

Given the evidence that that binding of HRGP to TSP-1 was mediated by the TSR, the same sequence motifs responsible for anti-angiogenic activity and CD36 binding, we explored the role of HRGP in regulating the anti-angiogenic activity of TSP-1. We found that binding of HRGP to TSP-1 abrogated the inhibitory effect of TSP-1 on EC migration and proliferation *in vitro* by interfering with TSP-1/CD36 interactions. We also demonstrated that HRGP inhibited the anti-angiogenic activity of TSP-1 *in vivo* in both the corneal angiogenesis and Matrigel plug model (50). HRGP also increased the angiogenic response to submaximal doses of bFGF in a CD36-dependent manner, suggesting that HRGP may have antagonized an endogenous, tonic CD36-dependent anti-angiogenic activity (figure 2).

6. THE TSP-1/CD36 ANTI-ANGIOGENIC PATHWAY IN TUMOR ANGIOGENESIS

6.1. Transcriptional regulation of TSP-1

There is considerable evidence that TSP-1 is an important mediator of the tumor suppressor effect of p53. Dameron *et al* showed that loss of wild type p53 in cultured fibroblasts from Li-Fraumeni patients correlated with reduced expression of TSP-1 and decreased ability to inhibit angiogenesis. In transfection assays, p53 stimulated the endogenous TSP-1 gene and positively regulated TSP-1 promoter sequences (51). In other studies, tumors over-expressing mutant p53 were shown to have a high micro-vessel density and decreased expression of TSP-1. Mice null for both p53 and TSP-1 also exhibited increased tumor growth in an osteosarcoma model (52). Similar regulation of TSP-1 has been demonstrated for PTEN and Smad4/DPC4 tumor suppressor genes. PTEN encodes a phosphatase with specificity for 3-phosphorylated inositol phospholipids. Inactivation of the PTEN gene has been detected in metastatic cancer and is associated with increased microvessel density and decreased TSP-1 expression. Reconstitution of wild-type PTEN in a murine glioma model decreased tumor growth, induced TSP-1 expression, and suppressed angiogenic activity. (53) The Smad4 tumor suppression gene is inactivated in gastrointestinal carcinomas and was characterized as a mediator of TGF-beta responses. The restoration of Smad4 expression in cell lines deficient for the gene suppressed tumor growth *in vivo* in nude mice, and was associated with increased TSP-1 expression and decreased angiogenesis.(54)

While tumor repressor genes appear to activate TSP-1, there is evidence that several oncogenes, including ras, c-jun, c-myc, and v-src inhibit TSP-1 expression (55). Watnick *et al* have further elucidated the pathways by which Ras expression downregulates TSP1, demonstrating that ras-dependent inhibition of TSP-1 expression is dependent on phosphorylation of c-myc. Recent studies by the same group showed that primary human mammary epithelial cells and human embryonic kidney cells expressing SV40 early region proteins, hTERT, and H-RasV12 were unable to induce a blood supply when injected into nude mice. This was shown to be due to ras-induced TSP-1 expression by the tumor cells (56).

Recent work on vascular development has also shed light on transcriptional regulation of TSP-1. Angiogenesis in the adult has long been thought to involve proliferation of EC derived from the local vasculature, and several groups have demonstrated that circulating EC progenitors derived from the bone marrow may participate in this process. Circulating bone marrow-derived EC progenitors have been shown to incorporate into new vasculature and proliferate (57)

The Id family of helix-loop-helix proteins play an important role in transcriptional regulation of cell growth and differentiation; in particular Id1 and Id3 have been shown to regulate angiogenesis in embryonic development and tumor growth. Using knockout mice, Lyden *et al* has shown that loss of a single Id1 or Id3 allele impairs metastasis and growth of tumor xenografts (58). Recent work has shown that TSP-1 is upregulated in Id1 null mice, leading to defects in angiogenesis. This suggests that Id1 functions as a repressor of TSP-1 transcription, and that regulation of angiogenesis by Id1 is mediated by TSP-1. Recent work has demonstrated that circulating endothelial progenitors are necessary for tumor angiogenesis and are recruited into the tumor vasculature in association with hematopoietic cells (59). Id mutant mice were transplanted with wild type bone marrow and tumor angiogenesis was restored through recruitment of bone marrow derived circulating EC precursors. The finding that TSP-1 is an effector of Id1 suggests that TSP-1 may serve to recruit CD36-positive endothelial cell precursors to tumor vasculature.

6.2. TSP-1/CD36 modulation in tumor models

The therapeutic potential of anti-angiogenesis therapy has been the focus of much attention. There is considerable evidence that TSP-1 is active in inhibiting tumor angiogenesis. Overexpression of TSP-1 in tumor xenografts inhibited tumor cell growth in a number of assays. (60, 61). Systemic treatment of mice with TSP-1 mimetic peptides inhibited tumor growth in murine models of bladder cancer and melanoma. TSP-1-derived peptides derived from the second type I repeat and containing D-isoleucine inhibited capillary EC growth and induced EC apoptosis, an effect that was blocked with antibody to CD36 (62). These data suggest that CD36 may be clinically useful target for antiangiogenic therapy.

In order to explore further the role of TSP-1 in tumor growth, Rodriguez-Manzaneque *et al* bred mammary tumor-prone mice carrying the neu/erbB2 oncogene under control of the mouse mammary tumor virus (MMTV) promoter with TSP-1-null and hTSP1 transgenic mice. They showed that tumor size and vasculature were significantly increased in TSP1-deficient mice, while in mice overexpressing TSP-1 tumors did not grow or grew more slowly. They went on to show that absence of TSP-1 resulted in higher levels of matrix metalloproteinase-9 (MMP9), a protease that promotes angiogenesis by digesting extracellular matrix releasing VEGF from extracellular stores (63). These studies suggest that although TSP-1 in tumors may play some role, TSP-1 in the matrix may be more important in inhibiting angiogenesis and tumor growth.

Our studies support a role for matrix TSP-1 in tumor angiogenesis, and suggest a role for HRGP in modulating TSP-1 activity. Using immunohistochemical studies of human breast cancer specimens, we showed that HRGP co-localized with TSP-1 in the tumor matrix, and that this interaction masked the anti-angiogenic epitope (TSR) of TSP-1 (50). We propose, therefore, that HRGP can interfere with the interaction of TSP-1 with CD36, and may therefore be a natural modulator of angiogenesis. The activity of HRGP may provide a mechanism by which tumors escape or become resistant to the anti-angiogenic effects of TSP.

6.3. Endothelial specificity of the TSP-1/CD36 anti-angiogenic system

Recent attention has focused on the unique properties of specific types of endothelium and the

characterization of lymphatic, large blood vessel, and microvascular endothelium has led to the search for selective angiogenesis factors. For example, the expression of the receptor tyrosine kinase VEGFR-3 (flt4) is restricted to the lymphatic endothelium in the adult, and may play a role in the regulation of "lymphangiogenesis," which may in turn be an important determinant of the pattern of lymphatic spread in cancer. (64) Patterns of CD36 expression may also influence lymphangiogenic responses. Hawighorst *et al* showed that immunostaining of murine squamous cell carcinoma and of human skin for CD36 revealed abundant expression of CD36 in blood vessels and little or no expression of CD36 in cutaneous lymphatic vessels. This group examined the role of TSP-1 in a multistep skin carcinogenesis model using transgenic mice with targeted overexpression of TSP-1 in the epidermis. They showed that TSP-1 overexpression inhibited the development of premalignant lesions and suppressed tumor angiogenesis but did not inhibit malignant conversion to squamous cell carcinoma, tumor-associated lymphangiogenesis or lymphatic tumor spread (65). These results suggest a distinct role for TSP-1 in blood vessel angiogenesis related to specific expression of CD36.

7. SUMMARY AND PERSPECTIVE

The growth and remodeling of new blood vessels involves the coordination of a complex set of pro- and anti-angiogenic factors. Dysregulation of angiogenesis may lead to pathologic conditions such as diabetic retinopathy, rheumatoid synovitis, and tumor growth, and defective angiogenesis may lead to impaired wound healing. TSP-1 was the first natural inhibitor of angiogenesis to be identified and has emerged as a major effector of angiogenesis inhibition. Transcriptional regulation of TSP-1 involves a number of tumor suppressor genes and oncogenes. CD36, which has been identified as the anti-angiogenic receptor for TSP-1, plays a critical role in modulating angiogenesis by transducing signals leading to EC apoptosis and blood vessel regression. *In vivo* models provide further evidence for the significant role of CD36 and TSP-1 in tumor angiogenesis. Further characterization of the structural properties, regulatory mechanisms, and signaling pathways of the CD36/TSP-1 anti-angiogenic pathway will enhance our understanding of these intricate processes and may provide attractive targets for therapeutic intervention.

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