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Regulation of nitric oxide signaling by thrombospondin-1: implications for anti-angiogenic therapies

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Abstract

In addition to long term regulation of angiogenesis, angiogenic growth factor signaling through nitric oxide (NO) acutely controls blood flow and hemostasis. Inhibition of this pathway may account for the hypertensive and prothrombotic side effects of vascular endothelial growth factor antagonists currently used for cancer treatment. The first identified endogenous angiogenesis inhibitor, thrombospondin-1, also controls tissue perfusion, hemostasis, and radiosensitivity by antagonizing NO signaling. We examine the role of these and other emerging activities of thrombospondin-1 in cancer. Clarifying how endogenous and therapeutic angiogenesis inhibitors regulate vascular NO signaling could facilitate development of more selective inhibitors.

The insight that tumor growth requires angiogenesis led to the development and clinical use of angiogenesis inhibitors as cancer therapeutics. The drugs bevacizumab, sorafenib, and sunitinib, which are approved by the US Food and Drug Administration for the treatment of several cancers, act specifically or in part by blocking the angiogenic activity of the vascular endothelial growth factor (VEGF) pathway. These targeted drugs significantly extend survival of cancer patients but have cardiovascular side effects that include hypertension[G] and thrombosis[G]^{1–3}. Although most attempts to define the etiology of their hypertensive activity have focused on long term changes in vessel architecture, VEGF signaling via nitric oxide (NO) also has acute effects on vessel tone[G]^{4,5}, and hypertension induced by the experimental VEGF receptor kinase inhibitor cediranib was recently shown to be caused by acute disruption of NO synthesis in vascular endothelium⁶. Recent studies of the first identified endogenous angiogenesis inhibitor, thrombospondin-1 (TSP1), reveal that it also inhibits NO-mediated signaling to acutely control tissue perfusion[G] and hemostasis[G]^{7,8}.

Interestingly, the pioneering work of Folkman and colleagues showed that tumors can produce circulating angiogenesis inhibitors⁹, and circulating TSP1 levels are elevated in people and mice with certain cancers^{10–12}. The benefit to the tumor of circulating angiogenesis inhibitors, which in some cases are produced by stromal rather than tumor cells, is unclear. We propose that elevated plasma TSP1 can enhance tumor perfusion through its hypertensive activity. This review synthesizes emerging evidence that hemostasis and tissue blood flow are acute targets of both endogenous and therapeutic angiogenesis inhibitors and explores ways that this insight can be used to improve anti-angiogenic therapy.

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Nitric oxide

Physiological activity of NO was first described by Davy in 1800¹³, but its production by mammalian tissues and role as a signaling molecule in vascular cells was not discovered until the 1980s¹⁴. The primary endogenous source of NO in endothelial cells is the endothelial isoform of nitric oxide synthase[G] (eNOS, also known as NOS3). eNOS is a highly regulated enzyme that is controlled by varying its expression, post-translational modification, subcellular localization, and binding of several regulatory proteins¹⁵. NO diffuses rapidly through tissue and across cell membranes and binds to its most sensitive known target soluble guanylate cyclase (sGC) to stimulate production of cGMP¹⁶, which regulates a number of signaling pathways that affect vascular cell function (Fig. 1a). NO at low concentrations promotes vascular cell survival, proliferation, and migration. Higher levels of NO directly or following conversion to other reactive nitrogen species trigger additional signaling pathways¹⁷, but the control of NO signaling in vascular cells appears to be specific for the NO/cGMP pathway, thus this is the focus of this Review¹⁸.

In the cardiovascular system NO/cGMP signaling has several important physiological functions (Fig. 1a). NO produced by endothelium diffuses into the vessel wall and relaxes vascular smooth muscle cells (VSMC) in arteries, thereby increasing vessel diameter, lowering resistance, and enhancing blood flow to tissues. Endothelial cell-derived NO thereby mediates acute local self-regulation of arterial tone in response to changes in mechanical shear[G] sensed by the endothelium and to circulating factors such as VEGF or acetylcholine[G]. NO also diffuses into the lumen of vessels, where it regulates hemostasis by limiting platelet aggregation¹⁹. Finally, NO acts in an autocrine manner to regulate endothelial permeability and angiogenesis²⁰. These vascular responses to NO occur over different time scales (Fig. 1b). Hemostasis and vessel tone are regulated acutely, whereas angiogenesis requires sustained elevation of NO levels.

Angiogenic factors stimulate NO synthesis

NO/cGMP signaling is required for angiogenesis in tumor and wound healing environments, and several angiogenesis factors stimulate this signaling node²⁰. VEGF activates eNOS through several pathways (Fig. 1c). The key role of eNOS in VEGF signaling is supported by deficiencies in both angiogenesis and acute permeability responses to VEGF in *eNOS*-null mice²¹. Angiogenic responses to angiopoietin-1 and angiopoietin-related factor are similarly deficient in *eNOS*-null mice^{22, 23}. Proangiogenic signaling by adrenomedullin, estrogens, insulin, lysophosphatidic acid, sphingosine-1-phosphate, and fibroblast growth factor-2 also involve NO^{15, 24–26}. Therefore, the NO/cGMP pathway is an important signaling node for a number of angiogenic factors.

Based on its important role in tumor angiogenesis, inhibition of eNOS has been considered as an approach to inhibit tumor growth. Accordingly, tumor growth rates and angiogenesis decrease in animals treated with NOS inhibitors²⁷, and angiogenesis and metastasis correlate with the level of eNOS expression²⁸. However, NO also has stimulatory and inhibitory roles in anti-tumor immunity²⁹. Inhibiting NOS delayed immune rejection of tumors³⁰, and stromal cell production of NO suppressed growth³¹. Therefore, general inhibition of NOS may not be an effective strategy to control tumor growth. At higher levels, NO is metabolized into reactive nitrogen species that have enhanced cytotoxic activities³².

What limits physiological NO signaling?

While the downstream biochemical and physiological targets of NO that mediate its positive effects on vascular cells are well understood, less attention has been given to negative regulation of NO/cGMP signaling. The distance NO diffuses in vascular tissues and the

duration of its signaling are limited by its binding to heme-containing proteins, especially to the abundant hemoglobin in red blood cells³³. NO bound to hemoglobin is converted to nitrite, which serves as a reservoir to regenerate NO under hypoxic conditions such as occur in solid tumors³³. Following metabolism to reactive nitrogen species, NO can also react with protein thiols or glutathione to form adducts that can regenerate nitrite or NO³⁴.

A family of cGMP phosphodiesterases play important roles in certain tissues to limit NO signaling by degrading the product of NO-driven sGC activation³⁵. Yet, the sustained physiological activity of inhaled NO gas^{36, 37}, the limited ability of phosphodiesterase inhibitors to dramatically alter blood pressure^{38, 39}, and the number of major human diseases associated with states of “NO insufficiency” of undefined origin^{40, 41} are compelling evidence that additional regulatory pathways must limit NO/cGMP signaling. One of these pathways is mediated by the secreted protein thrombospondin-1 (TSP1).

TSP1 blocks NO-driven angiogenesis

Thrombospondins are a family of 5 secreted proteins that play distinct roles in development and physiology⁴². TSP3–5 are pentameric proteins that are most abundant in bone and cartilage extracellular matrix. Specific mutations in TSP5 (cartilage oligomeric matrix protein) cause two inherited forms of dwarfism⁴³. To date, associations of TSP3–5 with cancer are limited to isolated reports of altered expression^{44–49}.

TSP1 and TSP2 constitute a subfamily of trimeric TSPs, and both are angiogenesis inhibitors⁵⁰. TSP1 is the most abundant protein in α -granules[G] of platelets, from which it is rapidly released during hemostasis⁵¹. Platelets may also serve as a buffer to control circulating TSP1 levels^{52, 53}. TSP2 is not found in platelets but is expressed by other vascular cells. Polymorphisms in *THBS1* and *THBS2*, which encode TSP1 and TSP2, have been associated with increased risk of cardiovascular disease^{54–56} but not to date with cancer⁵⁷.

Altered TSP1 and/or TSP2 expression has been observed in a number of human and murine cancers (for illustrative examples see Table 1). TSP1 expression in human cancers is inversely correlated with progression, and in a few cases was found to be an independent prognostic indicator^{58, 59}. One of the more consistent inverse relationships has been observed in colon cancer⁶⁰. However, lack of correlation with progression and a few positive correlations have also been reported.⁶¹ Careful examination of some tumors that showed a positive correlation, however, revealed that the increased TSP1 originated not from tumor cells but rather from stromal cells in the tumor^{62–64}. Induction of stromal TSP1 expression appears to be a paracrine effect of growth factors released by tumor cells. Studies of chemical-induced and spontaneous carcinogenesis in TSP1 or TSP2 transgenic mice support the hypothesis that paracrine induction of TSP1 and TSP2 expression plays an important inhibitory role in tumor progression^{65, 66}.

Loss of expression in cancers does not result from mutation or deletion of the *THBS1* and *THBS2* genes, so these are not classic tumor suppressors. Rather, expression is lost due to epigenetic silencing through hypermethylation^{59, 67, 68} or decreased by loss of positive regulation by tumor suppressors such as p53⁶⁹ or gain of negative regulation by oncogenes such as Ras, ID1, and MYC^{70–72}. Decreased levels of circulating TSP1 in certain inbred mouse strains are also associated with increased circulating endothelial precursors and susceptibility to cancers⁷³. Re-expressing TSP1 or TSP2 in cancers suppresses tumor growth and angiogenesis^{74–77}. Furthermore, recent evidence shows that TSP1 and TSP2 can also limit tumor growth by modulating innate anti-tumor immunity (Box 1).

The above findings have spurred efforts to identify active domains or peptides from TSP1 and TSP2 that could be developed as therapeutic inhibitors of angiogenesis and tumor growth⁵⁰,

^{78, 79}. A peptide sequence in the anti-angiogenic type 1 repeats of TSP1 was used to design the TSP1 mimetic ABT-510⁸⁰. This drug showed efficacy for cancer therapy in animal models and recently completed two phase II clinical trials^{81, 82}. ABT-510 also synergizes with chemotherapy in murine models⁸³, prompting the initiation of combination trials for cancer^{84–86}.

Despite clear evidence that TSP1 potently inhibits angiogenesis in vivo, relatively high concentrations of TSP1 are typically required to inhibit endothelial cell proliferation and migration in vitro^{87, 88}. One reason for this discrepancy is the absence of NO in such assays. TSP1 becomes a 100-fold more potent inhibitor in the presence of exogenous NO¹⁸. NO-stimulated vascular cell outgrowth into a 3D collagen matrix is dramatically greater in skeletal muscle explants from *Thbs1*-null compared to wild type mice. As little as 10 picomolar TSP1 blocks NO-stimulated increases in primary murine and human endothelial cell proliferation, matrix adhesion and migration. This concentration is less than that found in plasma⁵², indicating that circulating TSP1 levels are sufficient to limit physiological NO/cGMP signaling in endothelium.

TSP1 inhibits NO/cGMP signaling in other vascular cells

Inhibition of NO/cGMP signaling by TSP1 is not restricted to endothelial cells. TSP1 potently inhibits NO/cGMP signaling in VSMC and platelets^{8, 89}. As discussed below, VSMC are an important target through which TSP1 controls tissue perfusion. By suppressing the antithrombotic activity of NO, TSP1 released during platelet activation play an important role in hemostasis⁸. In contrast, TSP1 cannot regulate cGMP synthesis in most tumor cell lines⁹⁰, suggesting that inhibition of NO/cGMP signaling by TSP1 is specific to vascular cells.

TSP1 receptors mediating inhibition of NO/cGMP signaling

Two receptors (CD36 and CD47) mediate TSP1 inhibition of NO/cGMP signaling (Fig. 2). Vascular cells express at least 9 TSP1 receptors (Box 2)⁹¹, but initial studies focused on CD36 for inhibiting angiogenesis. Chemotaxis of CD36-deficient endothelial cells and corneal angiogenesis[G] in *CD36*-null mice were resistant to inhibition by TSP1^{92, 93}. Re-expression of CD36 in the endothelial cells restored inhibition by TSP1, indicating that this activity of TSP1 requires CD36 binding. Recombinant type 1 repeats of TSP1 that engage CD36, synthetic peptides derived from the same, and some CD36 antibodies mimic TSP1 to inhibit angiogenesis^{79, 92}. Consistent with these results, the same agents inhibited NO-stimulated responses in vascular cells^{8, 18, 89}. Thus, engaging CD36 is sufficient to inhibit NO/cGMP signaling. However, TSP1 remains a potent inhibitor of NO signaling in vascular cells lacking CD36⁹⁴. Therefore, CD36 is not necessary for TSP1 to inhibit NO/cGMP signaling. The corneal angiogenesis assay may over-emphasize the role of CD36 due to high concentrations of soluble VEGF receptor-1 that blocks VEGF signaling via NO in this tissue⁹⁵. Recent evidence suggests that the potent anti-angiogenic activity of ABT-510 may also be independent of CD36.⁹⁶

CD47 is the necessary receptor, based on the absence of TSP1 inhibitory activity in vascular cells from *CD47*-null mice⁹⁴. TSP1 engages CD47 via its C-terminal domain⁹⁷, and recombinant C-terminal domain and related CD47-binding peptides potently inhibit NO signaling⁹⁴. Thus, TSP1 blockade of physiological NO-signaling in vascular cells requires CD47. CD36 engagement is sufficient to activate the inhibitory signal, but only in cells that also express CD47⁹⁴. Furthermore, 100-fold lower concentrations of TSP1 are sufficient to inhibit NO signaling in vascular cells via CD47 versus CD36 (Fig. 2). The physiological importance of endogenous TSP1/CD47 signaling is shown by the elevated basal cGMP levels in vascular cells and muscle tissue from *Thbs1*- and *CD47*-null mice, which *CD36*-null cells lack^{94, 98}.

TSP1 inhibits NO/cGMP signaling at multiple levels

sGC is clearly one target of inhibitory TSP1 signaling through CD47 (Fig. 2)^{91, 99}. In addition to activation by NO binding to its heme moiety, sGC can be activated by post-translational modifications, drugs, and interactions with activating proteins¹⁰⁰, but the signal transduction pathway through which CD47 ligation by TSP1 inhibits sGC activation is currently unknown. It will be important to determine whether this inhibitory signaling extends to pharmacological activators of sGC to assess whether pathological elevation of TSP1 levels would obviate the activity of sGC-activating drugs currently under development for treating cardiovascular diseases associated with NO-insufficiency¹⁰¹.

Remarkably, TSP1 also inhibits functional responses driven by 8Br-cGMP, a cell-permeable analog of cGMP, including a delay in platelet aggregation¹⁸. TSP1 inhibits 8Br-cGMP-stimulated phosphorylation of platelet VASP[G] at Ser²³⁹, a direct target of cGMP-dependent protein kinase (cGK)¹⁰². 8Br-cGMP-induced phosphorylation of a defined cGK peptide substrate in platelet lysates is inhibited by pretreatment of platelets with TSP1. Therefore, cGK is a second target of inhibitory TSP1 signaling (Fig. 2).

Further studies revealed a third site at which TSP1 controls physiological NO signaling. Extracellular myristate stimulates eNOS activity in a CD36- and AMP kinase-dependent manner¹⁰³ (Fig. 2). CD36 is a known transporter of free fatty acids¹⁰⁴, and TSP1 and a CD36 antibody known to mimic its anti-angiogenic activity potently block radiolabeled myristate uptake via CD36 into endothelial cells¹⁰⁵. TSP1 blocks myristate-stimulated cGMP synthesis in an eNOS-dependent manner. Thus, inhibition of myristate uptake via CD36 and consequent blocking of eNOS activation is a third mechanism by which TSP1 limits NO/cGMP signaling in vascular cells (Fig. 2). Because 1–10 nM TSP1 is required to inhibit myristate uptake, whereas 10 pM TSP1 is sufficient for blocking sGC and cGK activation via CD47, the latter targets should be dominant for cell-autonomous inhibition of NO signaling. However, the ability to inhibit endothelial NO synthesis through CD36 provides a mechanism by which circulating TSP1 could also limit the paracrine effects of endothelium-produced NO on platelets and VSMC (Fig. 1a).

Do other TSPs inhibit NO/cGMP signaling?

Given the potent and redundant regulation of vascular NO/cGMP signaling by TSP1, it is important to know whether this activity is shared by other members of the TSP family. All 5 TSPs have conserved sequence motifs in their C-terminal G-module that are known to bind to CD47 as synthetic peptides (Fig. 2)¹⁰⁶, and one study provided correlative evidence that both TSP1 and TSP2 regulate inflammatory responses via CD47¹⁰⁷. However, we found that the C-terminal signature domains[G] of TSP2 and TSP4 bind much less avidly to CD47-expressing cells than does the signature domain of TSP1¹⁰⁸. Consistent with their decreased binding avidities, the signature domains of TSP2 and TSP4 are less active for inhibiting NO-stimulated cGMP synthesis in vascular cells. Therefore, potent antagonism of NO signaling is relatively specific for TSP1. As discussed below, this selectivity is reflected in several phenotypes of *Thbs1*- versus *Thbs2*-null mice.

TSP1 acutely regulates blood flow

The activity of TSP1 via CD47 to block NO-stimulated relaxation of contracting VSMC in vitro⁸⁹ translates directly to regulation of blood flow in vivo. Challenging *Thbs1*- or *CD47*-null mice with NO results in twice the increase in regional blood flow as in wild type animals⁷. Thus, endogenous TSP1 engaging CD47 constantly limits the dynamic blood flow response of healthy tissue to NO.

Antagonism of NO/cGMP signaling by TSP1 becomes more pronounced under ischemic conditions. This is relevant to cancer because of the transient and persistent hypoxic conditions found in solid tumors¹⁰⁹. Ischemic soft tissue survival and blood flow are significantly enhanced in mice lacking TSP1 or CD47. This is true for partial fixed ischemia[G] models employing skin flaps or femoral artery ligation in hindlimbs and for total ischemia[G] in full thickness skin grafts^{7, 110, 111}. Conversely, loss of tissue blood flow and tissue necrosis in wild type mice can be prevented by blocking TSP1/CD47 signaling using antibodies or antisense techniques^{98, 99, 110, 111}. Consistent with its weak binding to CD47¹⁰⁸, *Thbs2*-null mice show no advantage in the same ischemic injury models¹⁰⁸, further indicating that this is a specific function of TSP1.

Reperfusion of ischemic tissue results in an additional stress mediated by recruitment and activation of immune inflammatory cells. These in turn release inflammatory mediators and reactive oxygen species, yielding an ischemia-reperfusion (I/R) injury¹¹². Increasing NO decreases I/R injury by improving return of blood flow and limiting inflammatory cell activation. Following liver I/R, *Thbs1*- and *CD47*-null mice show better preservation of liver cytoarchitecture and lower serum levels of released liver enzymes than wild type mice¹¹³. Furthermore, pre-treating wild type animals with a function-blocking CD47 antibody decreases liver I/R injury. Therefore, endogenous TSP1 augments both fixed ischemic and I/R injury responses. Given that solid tumors are constantly subject to cycles of I/R¹⁰⁹, the loss of TSP1 expression in some tumors could offer a selective advantage by maximizing the anti-inflammatory activity of NO produced in the tumor.

Does flow regulation select for loss of TSP1 in cancer?

Although TSP1 clearly has acute effects on tissue perfusion in addition to its long term regulation of angiogenesis, it was unclear whether such acute regulation extends to blood flow in tumors. On the one hand, the tumor vasculature is anatomically abnormal¹¹⁴. The tortuous channels and poor organization of tumor vasculature creates turbulent flow and transient interruptions in perfusion, both of which increase overall resistance and limit the degree to which flow could increase in response to dilation of arteries and arterioles feeding the tumor vascular bed. The characteristic leakiness of tumor vasculature also results in increased interstitial pressure¹¹⁵, which further limits the ability of tumor perfusion to increase in response to increased pressure in the perfusing vessels following upstream vasodilation by NO.

An ability to acutely regulate the perfusion of tumors is desirable for cancer treatment, for example to enhance hyperthermia therapy[G] by decreasing tumor blood flow, or to increase intravenous delivery of cytotoxic therapeutics by increasing flow. In many but not all tumors, vasodilators such as NO diminish flow through the tumor, while vasoconstrictors increase flow^{116, 117}. This paradoxical outcome has given rise to the concept of a “vascular steal phenomenon,”¹¹⁸ which is based on evidence that healthy tissue is more responsive to vasoactive agents than are vessels in the tumor (Fig. 3a). Thus, systemic vasodilation would “steal” blood flow from the tumor by selectively decreasing resistance in the healthy tissues.

We recently examined the ability of TSP1 to acutely regulate tumor blood flow using syngeneic melanomas grown in wild type and *Thbs1*-null mice⁹⁰. Blood flow in tumor versus normal tissue was imaged using blood oxygen level-dependent (BOLD)-MRI[G]. Consistent with BOLD-MRI data in healthy mice at rest⁷, flow through normal soft tissue increased when tumor-bearing mice were challenged using a rapidly releasing NO donor, and the increase was greater in *Thbs1*-null mice. In contrast, flow through the tumor decreased immediately following NO challenge and did not differ between the *Thbs1*-null and wild type mice. This decrease in tumor flow is consistent with a steal effect, indicating that the tumor vasculature is less able to acutely dilate in response to NO. Because tumors in wild type and *Thbs1*-null

mice showed similar resistance, this inability to respond to NO is not dependent on TSP1 expression in the tumor vasculature. Therefore, the tumor vasculature has a limited ability to acutely alter its perfusion, due to either its abnormal vascular architecture or an absence of functional smooth muscle¹¹⁹. Rather, vasoactive agents indirectly alter tumor perfusion by either increasing or decreasing the hydrostatic pressure in major vessels supplying the tumor (Fig. 3a).

Acute flow changes following vascular challenge were also examined in a melanoma xenograft model⁹⁰. The parental melanoma cell line MDA-MB-435 lacks significant TSP1 expression and is tumorigenic in athymic mice⁷⁴. Like the syngeneic melanoma, NO challenge decreased flow through this xenograft tumor while increasing flow in the contralateral leg, and injection of the vasoconstrictor epinephrine increased flow through the tumor while decreasing flow elsewhere. However, when the same tumor cells were engineered to express TSP1, which decreases growth, angiogenesis, and spontaneous lung metastasis⁷⁴, the acute decrease in flow following NO challenge was diminished, and epinephrine challenge did not increase flow. Therefore, TSP1 secretion by a tumor can limit its acute responsiveness to blood flow regulation. This could involve local effects of TSP1 on the tumor vasculature, but a more likely explanation is that TSP1 released into the circulation from the tumor tempers acute responses of vascular beds outside of the tumor to vasodilator or vasoconstrictor challenge (Fig. 3b).

The concept that TSP1 can temper acute regulation of tumor blood flow has several implications. Although TSP1 expression is frequently suppressed in tumors, elevated circulating TSP1 levels derived from stromal cells have been reported in certain human cancers^{11, 12}. Our data suggest that such increased circulating TSP1 could increase tumor perfusion if it selectively vasoconstricts and thereby limits perfusion of healthy tissues. This hypothesis is consistent with the known ability of endogenous TSP1 to limit blood flow through normal muscle and in ischemic tissues⁷. A second implication of this data concerns the use of angiogenesis inhibitors in conjunction with chemotherapy or radiotherapy. To enhance radiotherapy and chemotherapy responses, agents have been sought that temporarily increase tumor perfusion. Our results suggest that a TSP1 mimetic administered prior to irradiation could temporarily increase tumor perfusion and thereby enhance responses to radiotherapy. Similarly, TSP1 mimetics could improve tumor delivery of chemotherapeutics by selective vasoconstriction of extratumoral vessels (Fig. 3b). However, such mimetics should be used with caution since they would be expected to also have similar prothrombotic activity as TSP1⁸. Indeed, we found that ABT-510 is a weak inhibitor of myristate uptake via CD36 (Fig. 2), and ABT-510 has corresponding prothrombotic activities in platelets⁹⁶ and some patients⁸⁶.

Do therapeutic angiogenesis inhibitors resemble TSP1?

Analogous to TSP1 and ABT-510, the hypertensive activity of therapeutic angiogenesis inhibitors may contribute to their efficacy. Bevacizumab, sunitinib, and sorafenib block VEGF-dependent activation of eNOS (Fig. 4). In addition to inhibiting NO/cGMP signaling in endothelial cells, these inhibitors decrease the flux of NO released by endothelial cells to act on other vascular targets. This can explain the acute regulation of blood pressure by intravenous VEGF and cediranib⁶. By analogy, we argue that the prothrombotic and hypertensive activities reported for clinical angiogenesis inhibitors have a similar basis (Fig. 4; Table 2). A similar explanation was recently proposed for the renal side effects of these drugs¹²⁰.

TSP1 and cancer evolution

The elevated circulating TSP1 levels typical of certain cancers may provide a selective advantage to the tumor by maximizing tumor perfusion at the expense of other vascular beds. If circulating TSP1 in cancer does serve to increase perfusion of the tumor, could the discovery

of other circulating angiogenesis inhibitors such as endostatin⁹, which inhibits eNOS activation via PP2A¹²¹ and inhibits sGC expression¹²² (Fig. 4), indicate that their release into the host circulation generally confers an advantage to the tumor by increasing perfusion? This could be viewed as a competitive evolutionary adaptation of tumors to maximize the nutrients they can obtain¹²³. Conversely, platelet interactions with tumor cells play a critical role in metastasis¹²⁴, so the activity of TSP1 to increase platelet adhesion⁸ could provide a selective advantage to account for the observed increased metastatic spread of some tumors that induce higher levels of circulating TSP1¹²⁵. Both of these hypotheses merit further study to define what selective pressures drive these changes in TSP1 expression.

TSP1 and radiosensitivity

Angiogenesis inhibitors are increasingly being tested in combination with radiotherapy. Empirical evidence from animal tumor models and human clinical trials indicates that this combination is beneficial¹²⁶, but the mechanism remains controversial. On the one hand, tumors have hypoxic regions, and based on their ability to “normalize” the tumor vasculature, angiogenesis inhibitors may improve the overall oxygenation of tumors in an NO-dependent manner¹²⁷. Increased tissue oxygenation can increase radiosensitivity^{109, 128}. This scenario predicts that prior treatment with angiogenesis inhibitors would produce the best enhancement. However, some studies have shown enhanced radiosensitivity when angiogenesis inhibitors are administered either immediately before or after irradiation¹²⁹. In this case, mechanisms other than vascular normalization must account for their efficacy. Based on the radioresistance of tumors grown in mice lacking Bax or acid sphingomyelinase, which protect the tumor vasculature from radiation-induced apoptosis, tumor endothelial cells were proposed to be the critical radiosensitive component of tumors¹³⁰. Thus, combining angiogenesis inhibitors with radiation could enhance apoptosis within the tumor vasculature and thereby starve the tumor of nutrients. This could account for some acute radiosensitization by angiogenesis inhibitors. However, other studies have questioned the limiting role of tumor vasculature in tumor radioresistance^{131, 132}.

Given the many variables that can influence tumor tissue responses to radiation, it is important to first understand how angiogenesis inhibitors influence radiosensitivity in normal tissue. In addition to shedding light on cancer radiosensitivity, any manipulation that improves radioresistance of normal tissue could be exploited to locally protect surrounding healthy tissue and thereby maximize the radiation dose that can be delivered to an underlying tumor. If tumor radiosensitivity indeed proves to be more important than that of the tumor vasculature, systemic administration of a vascular radioprotectant could prevent radiation damage to healthy tissue without compromising tumor ablation.

Assessing the radiosensitivity of healthy soft tissue in mice, we recently found that *Thbs1*-null mice are remarkably resistant to high dose (25 Gy) hindlimb irradiation¹³³. In the skin, alopecia [G] and wet desquamation [G] were decreased compared to that observed in wild type mice, and histological examination after 2 months confirmed preservation of skin architecture and function. Remarkably, underlying skeletal muscle in *Thbs1*-null mice showed essentially no signs of necrosis or fibrosis following irradiation. Not only was muscle mitochondrial function preserved, but the null mice tended to have greater muscle mass in the irradiated hindlimb compared to nonirradiated control limbs. Responses to irradiation in *CD47*-null mice paralleled those of the *Thbs1*-null, indicating that this TSP1 receptor is critical to the radiosensitizing activity of endogenous TSP1. *Thbs2*-null mice showed no protection from radiation injury, however, indicating that this activity is specific for TSP1.

Shortly following irradiation, apoptosis was also decreased in muscle and bone marrow of *Thbs1*- and *CD47*-null mice. Because CD47 and TSP1 are also implicated in recruitment of

inflammatory cells at sites of infection or injury^{97, 134}, we considered whether the apparent radioprotection could be secondary to a decreased inflammatory response in the irradiated null limbs. To eliminate this variable, radiosensitivity was assessed in vascular cells isolated from the respective mice. Remarkably, *Thbs1*- and *CD47*-null cells in culture replicated the radioresistance of their hosts¹³³. The null cells exhibited less death following irradiation, and over time in culture, maintained proliferative capacity that was progressively lost by irradiated wild type cells. Thus, eliminating the signal from secreted TSP1 engaging its receptor CD47 dramatically protects the viability and proliferative capacity of cells following high dose irradiation. Cell-autonomous radioprotection in the null cells indicates that blocking this pathway in vivo could be protective independent of any effect of these proteins on the immune system. Although elevating NO is known to be radioprotective for whole body irradiation¹³⁵, ongoing experiments indicate that regulation of NO/cGMP signaling only partially explains this radioprotection. Additional molecular mechanisms are under investigation.

Returning to the controversy concerning the relative contribution of tumor cells versus host vasculature to radiosensitivity, we compared radiation growth delays for syngeneic B16 melanomas implanted in wild type versus *Thbs1*-null mice¹³³. No decrease in irradiation-induced regrowth delay was observed in the *Thbs1*-null mice. Therefore, antagonists of TSP1/CD47 interaction merit testing as selective radioprotectants and could permit higher radiation doses to be delivered to tumors while preserving critical adjacent tissue and organ function.

Conclusions and perspectives

Several common themes are emerging from studies of the endogenous inhibitor TSP1 and therapeutic angiogenesis inhibitors. First, we must not allow the name “angiogenesis inhibitor” to limit how we view the physiological pathways influenced by these agents. NO/cGMP signaling is an important component of pro-angiogenic signaling downstream of VEGF and other vascular growth factors, but the same pathway also mediates acute effects of VEGF signaling on vascular smooth muscle tone and regulates platelet homeostasis (Fig. 4). Just as VEGF has both long term and acute effects on vascular physiology and thrombosis, endogenous and therapeutic angiogenesis inhibitors should be expected to acutely affect the same pathways. TSP1 is an acute vasoconstrictor¹³⁶, and bevacizumab and VEGFR kinase inhibitors exhibit similar hypertensive activities. TSP1 and the therapeutic VEGF pathway antagonists also share similar prothrombotic activities. Because NO rapidly diffuses between cells, blocking NO synthesis by endothelial cells using VEGF pathway antagonists lowers the level of NO seen by platelets and VSMC.

This concept is beginning to be recognized in attempts to overcome the side effects of therapeutic VEGF antagonists⁶. If NO/cGMP signaling is critical for both angiogenesis and acute cardiovascular regulation, then manipulation of other pathways unique to acute vascular regulation may compensate for loss of NO signaling. Thus, use of antihypertensive drugs (an angiotensin-converting enzyme inhibitor or a calcium channel blocker) overcame the hypertensive activity of the VEGF signaling inhibitor cediranib without blocking its inhibition of tumor growth^{6, 120}.

What does this teach us about designing a better angiogenesis inhibitor? One scenario would be to selectively target components of the NO/cGMP signaling cascade that mediate long term regulation of angiogenesis but not acute cardiovascular responses. The current FDA-approved angiogenesis inhibitors all are expected to inhibit eNOS activation and thus lower the level of NO available for its paracrine regulation of VSMC and platelets. TSP1 signaling via CD47 inhibits sGC and cGK activation. Drugs that selectively inhibit at this level in endothelium could potentially block the autocrine pro-angiogenic activity of NO without blocking physiological NO paracrine signaling to the underlying VSMC. Antibodies that engage CD47

and mimic TSP1 binding would be one such approach. These would engage CD47 on the endothelium but be too large to cross the basement membrane to inhibit signaling in the VSMC. CD47 antagonists may also provide an advantage over VEGF antagonists by their ability to also block NO/cGMP signaling induced by other tumor-derived angiogenic factors (Fig. 4).

As we learn more about the specific downstream targets of NO/cGMP signaling that are critical for long term angiogenic responses versus acute vasoregulation and hemostasis, additional targets may be identified that can selectively block the pro-angiogenic aspects of NO/cGMP signaling. Achieving this goal will require further efforts to map the signaling networks mediating acute and chronic responses to NO in each vascular cell type.

Studies of TSP1 are also teaching us that angiogenesis inhibitors can profoundly affect other aspects of tumor biology including anti-tumor immunity and radiotherapy responses. Although TSP1 potently inhibits NO/cGMP signaling, additional signaling pathways controlled by TSP1 may be important for these other activities. Investigating these may identify additional targets for controlling tumor growth and improving responsiveness to ablative therapies.

Display items

Box 1

Expanding the role of TSP1 in cancer to innate immunity[G]

In addition to suppression of angiogenesis and acute blood flow regulation, down-regulation of TSP1 and TSP2 during tumor progression and inhibition of growth by re-expression of the proteins in murine models can also result from modulation of innate anti-tumor immunity¹³⁷. Over-expression of TSP1 in melanoma cells causes delayed growth and increased tumor-associated macrophage (TAM) recruitment into xenograft tumors grown in nude mice⁷⁴. However, TAM can differentiate into either cytotoxic (M1) or tumor growth-promoting (M2) states. Increased iNOS is a hallmark of M1 macrophages¹³⁸, and although M1 cytotoxic macrophages are a minor fraction of the TAM in MDA-MB-435 tumors, their recruitment or differentiation is increased when those tumors express TSP1. *In vitro*, TSP1 acutely induces expression of PAI-1, an important regulator of macrophage migration^{139, 140}, by human and mouse macrophages. PAI-1 is strongly expressed by TAM in TSP1-overexpressing tumors *in vivo*, suggesting that TSP1-induced macrophage recruitment is at least partially mediated by PAI-1¹³⁷. Moreover, TSP1 and TSP2 cause a significant increase in phorbol ester-mediated superoxide generation by M1-differentiated human monocytic cells, which mediates tumor cell killing, by interacting with $\alpha 6 \beta 1$ integrin through their NH₂-terminal domains. TSP1 stimulates killing of breast carcinoma and melanoma cells by M1-polarized macrophages *in vitro* via this release of ROS. Taken together, these data suggest that TSP1 plays an important role in innate anti-tumor immunity by enhancing recruitment and activation of M1 TAM. Thus, avoiding this innate immune surveillance could provide an additional selective pressure for loss of TSP1 and TSP2 expression during tumor progression.

iNOS, inducible nitric oxide synthase; PAI-1, plasminogen activator inhibitor-1;

ROS, reactive oxygen species; TAM, tumor-associated macrophages.

Box 2

TSP1 receptors that regulate angiogenesis

Although intact TSP1 is a potent angiogenesis inhibitor, its N-terminal domains have pro-angiogenic activities^{141, 142}. These are mediated by interactions with several TSP1-binding

integrins: $\alpha 3\beta 1$, $\alpha 4\beta 1$, $\alpha 6\beta 1$, and $\alpha 9\beta 1$ ^{141, 143–145}. The N-terminal domain of TSP1 also modulates endothelial cell focal adhesions and vascular permeability by engaging calreticulin and low density lipoprotein receptor-related protein-1^{146, 147}.

The central type 1 repeats of TSP1 and TSP2 inhibit angiogenesis by engaging the receptor CD36^{92, 148}. CD36, a class B scavenger receptor and fatty acid translocase¹⁰⁴, is expressed on microvascular endothelium and VSMC. Peroxisome proliferator-activated receptor- γ ligands increase vascular expression of CD36 and correspondingly increase tumor responses to the CD36-targeted drug ABT-510¹⁴⁹. A coding polymorphism in CD36 was associated with increased risk of colorectal carcinoma in a Japanese population¹⁵⁰.

CD47 is a high affinity cell surface receptor for the C-terminal domain of TSP1 and a low affinity receptor for this domain of TSP2¹⁰⁸, but the specific residues in TSP1 that mediate binding to CD47 is currently a subject of debate^{151–153}. CD47 is ubiquitously expressed and also plays an important role in regulating phagocytosis through engaging its counter-receptor SHPS1¹⁵⁴. In some hematologic malignancies and breast cancer, antibody targeting of CD47 can kill tumor cells^{155, 156}.

At-a-glance

- Nitric oxide (NO) mediates pro-angiogenic activities of vascular endothelial growth factor and a number of additional angiogenic factors that support the neovascularization of tumors. This signaling involves activation of soluble guanylate cyclase and downstream targets of cGMP.
- Thrombospondin-1 (TSP1) and TSP2 are endogenous angiogenesis inhibitors. Their expression is frequently lost during cancer progression, and over-expression suppresses tumor growth. TSP1 is a potent antagonist of NO signaling in vascular endothelial cells, vascular smooth muscle cells, and platelets. TSP2 is substantially less active.
- Antagonism of NO signaling can be mediated by TSP1 binding to two of its receptors, CD36 and CD47, but CD47 mediates inhibition at much lower concentrations of TSP1, and CD47 is also required for signaling through CD36.
- TSP1 signaling through CD47 redundantly inhibits NO signaling at the level of soluble guanylate cyclase and cGMP-dependent protein kinase.
- In addition to long term inhibition of angiogenesis, antagonism of NO signaling by TSP1 results in acute inhibition of tissue perfusion and acceleration of platelet hemostasis. Consequently, TSP1 and CD47 limit recovery of blood flow and tissue survival following ischemic injuries. In contrast, tumor vasculature is resistant to acute vasodilation by NO, and increased circulating TSP1 produced by tumor stroma may instead indirectly increase tumor perfusion by limiting blood flow elsewhere.
- These activities of TSP1 parallel the hypertensive and prothrombotic activities that are emerging as frequent side effects of therapeutic angiogenesis inhibitors, and both can be explained by inhibition of NO signaling.
- TSP1 signaling through CD47 also limits soft tissue survival after radiation injury. Disruption of this pathway is a possible strategy to permit delivery of higher therapeutic radiation doses to tumors while selectively protecting surrounding healthy tissues.

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Glossary

Hypertension	A condition in which blood pressure is chronically elevated
Thrombosis	Formation of a blood clot (thrombus) inside a blood vessel that obstructs blood flow
Vessel tone	An assessment of vessel resistance to blood flow. Vessel tone is decreased by NO-mediated vasodilation and increased by activity of autonomic innervation and the presence of vasopressors
Tissue perfusion	The volume of blood that flows through a unit quantity of tissue
Hemostasis	Physiological process that arrests bleeding at a site of injury by formation of a hemostatic plug, resolves this plug during healing, and maintains circulating blood in a fluid state in the absence of injury
Nitric oxide synthase	Mammalian genomes encode three nitric oxide synthases: NOS1 or nNOS is constitutively expressed in neuronal tissues, NOS2 or iNOS is an inducible enzyme in phagocytic cells that can also be induced by inflammatory mediators in vascular cells, and NOS3 or eNOS is a constitutively expressed but highly regulated enzyme in endothelium and platelets
Mechanical shear	A physical force exerted on endothelial cells in blood vessels as a result of blood flow, which regulates the activity of eNOS in endothelium
Acetylcholine	A neurotransmitter in the autonomic nervous system that activates eNOS in endothelial cells by increasing cytoplasmic calcium levels
α -Granules	A major secretory organelle in platelets. The primary source for purification of thrombospondin-1
Corneal angiogenesis assay	A standard method to assess pro- and anti-angiogenic factors by implanting slow-releasing polymer pellets containing the factors into the avascular cornea, usually of mice, rats, or rabbits
VASP	Vasodilator-stimulated phosphoprotein is a protein that controls actin polymerization. It is a well characterized substrate for phosphorylation by cGMP-dependent protein kinase
Signature domain	Conserved C-terminal region of thrombospondins that includes EGF-like repeats, calcium-binding repeats and the C-terminal globular domain. Contains the binding site for CD47 in TSP1
Partial fixed ischemia	A partial but sustained loss of tissue blood flow
Total ischemia	Sustained total loss of tissue blood flow
Hyperthermia therapy	The use of heat to damage or kill cancer cells and to make cancer cells more sensitive to the effects of radiation and certain anticancer drugs
Blood oxygen level-dependent magnetic	An imaging technique that assesses blood oxygenation by detecting oxygen bound to hemoglobin

resonance imaging (BOLD-MRI)

Alopecia	A temporary or permanent loss of hair. Typically occurs several weeks following high dose irradiation for tumor treatment
Wet desquamation	Skin damage leading to broken skin or infection due to a fluid filled abscess created by a blocked ducts or capillaries. A characteristic side effect of high dose irradiation
Innate immunity	Cells and mechanisms that defend the host from pathogens or tumors in a non-specific manner. Innate anti-tumor immunity involves primarily macrophages and natural killer cells

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a

b

Fig. 1c

Figure 1. The central role of nitric oxide (NO) signaling in angiogenesis, vascular tone, and hemostasis

a | Vascular endothelial growth factor (VEGF) binding to its receptor on endothelial cells activates nitric oxide synthase (eNOS) to produce the diffusible signaling molecule NO. NO acts in an autocrine manner to stimulate endothelial cell growth and motility leading to angiogenesis. VEGF signaling via NO also contributes to increasing vascular permeability. NO diffuses into vessel walls, causing arterial vessels to relax and increase blood flow. NO also acts in a paracrine manner to prevent thrombosis by inhibiting platelet adhesion and aggregation. **b** | Different vascular activities of NO occur on different time scales. **c** | In endothelial cells, VEGF signaling through VEGFR2 activates the phosphatidylinositol 3-kinase (PI3K) pathway; Akt then phosphorylates human eNOS at Ser¹¹⁷⁷, Ser¹⁵⁷, Ser¹⁵⁸, activating eNOS and decreasing its calcium dependence. The kinase Src, which is activated by VEGF, also activates eNOS through two mechanisms: phosphorylation of Tyr⁸³, Ser¹⁵⁹ and phosphorylation of heat shock protein 90 (Hsp90), which then binds to eNOS and activates NO synthesis¹⁶⁰. Simultaneously, VEGFR2 signaling through phospholipase-Cγ (PLCγ) mobilizes intracellular Ca²⁺, which further activates eNOS in a calmodulin (CaM)-dependent manner, and increases AMP kinase (AMPK)-mediated eNOS phosphorylation at Ser¹¹⁷⁷¹⁶¹. NO produced by eNOS binds to the prosthetic heme on soluble guanylate cyclase (sGC) to stimulate cGMP synthesis, activating cGMP-dependent protein kinase (cGK-I) and cGMP-gated channels to regulate downstream targets that increase endothelial cell proliferation, migration, survival, and permeability¹⁴. Additional parallel signaling through Src, Akt, and the protein kinase C-mitogen-activated protein kinase pathway (PKC-Raf1-MEK-ERK) synergizes with NO/cGMP signaling to support each of these endothelial cell responses. In VSMC and platelets, the same downstream pathways are activated by exogenous NO diffusing from endothelium.

Figure 2. CD47 and CD36 mediate inhibition of NO/cGMP signaling by thrombospondin-1

Thrombospondin-1 (TSP1) redundantly inhibits cGMP signaling driven by exogenous or endogenous NO in vascular cells via 2 receptors. The C-terminal G-module of TSP1 contains two peptide motifs that have been shown to bind CD47¹⁰⁶. The yellow highlighted residues in these peptides are conserved across all 5 TSPs, but studies using recombinant domains of TSP2 and TSP4 indicate that high affinity binding to CD47 is specific to TSP1¹⁰⁸. Binding of TSP1 or the peptides signals via CD47 to inhibit both NO-mediated activation of soluble guanylate cyclase (sGC) in endothelial cells, VSMC, and platelets and cGMP-mediated activation of cGMP-dependent protein kinase (cGK-I) in platelets. At elevated levels, TSP1 also interacts with CD36 via its central type 1 repeats and thereby inhibits its fatty acid translocase activity¹⁰⁵, limiting myristate uptake into the cytoplasm and thereby inhibiting membrane translocation of Src family kinases in endothelial cells. CD36-dependent myristate uptake activates two known eNOS kinases: Src and AMP kinase (AMPK)^{96, 103}, but the relative importance of these two pathways in controlling eNOS activation through CD36 has not been established. The TSP1 mimetic drug ABT-510 also binds to CD36 and inhibits angiogenesis via both translocase-dependent and caspase-dependent mechanisms^{96, 162}.

Fig. 3a

Fig. 3b

Figure 3. Vascular responses outside the tumor primarily determine effects of TSP1 on tumor perfusion

a | Tumor vasculature is much less responsive than normal vasculature to physiological regulation by vasodilators such as NO and vasoconstrictors such as epinephrine. Thus, vasodilation indirectly decreases blood flow through the tumor, and vasoconstriction increases flow through the tumor. **b** | These responses to vasoactive agents are not sensitive to endogenous TSP1 in the tumor vasculature, but over-expression of TSP1 in the tumor either locally or by release into the circulation tempers responses to vasoactive agents in the host vasculature and decreases the “steal” phenomenon in mice bearing TSP1-expressing tumors. The relative contribution of local versus systemic effects of TSP1 on tumor vascular resistance remains to be defined.

Figure 4. Convergence of angiogenesis inhibitor signaling on the nitric oxide (NO)/cGMP cascade
The current US Food and Drug Administration (FDA)-approved angiogenesis inhibitors for cancer therapy block vascular endothelial growth factor (VEGF) binding to the VEGF receptor (VEGFR2) (the inhibitor bevacizumab) or inhibit the tyrosine kinase activity of VEGFR2 (the inhibitors sorafenib and sunitinib). VEGFR2 activates several parallel signaling pathways that control angiogenesis, but inhibiting NO/cGMP signaling blocks most of these responses. Therapeutic responses to VEGF inhibitors may be limited by parallel activation of the downstream pathways by additional angiogenic factors produced by tumors that can activate NO/cGMP signaling independent of the VEGF receptor such as adrenomedullin, angiopoietin-1, sphingosine 1-phosphate (S1P), lysophosphatidic acid (LPA), angiopoietin-related growth factor (AGF), estrogens, insulin, and fibroblast growth factor-2 (FGF2)^{15, 22–24}. Therefore, inhibitors that act on essential downstream pathways may be more effective than the existing VEGF/VEGFR antagonists for controlling tumor angiogenesis. Endostatin is one such agent that acts via the phosphatase PP2A to inhibit nitric oxide synthase (eNOS) activation¹²¹ and downstream to inhibit soluble guanylate cyclase (sGC) expression¹²². Thrombospondin 1 (TSP1) is a redundant inhibitor of the NO/cGMP cascade, and the experimental drug ABT-510, a TSP1 mimetic, inhibits angiogenesis via the TSP1 receptor CD36⁹⁶. However, native TSP1 also acts through the more potent inhibitory pathway mediated by CD47. Proangiogenic factors and signaling pathways are shown in green, and anti-angiogenic factors and pathways are in red.

Table 1

Thrombospondin expression and cancer progression*

Study Design	Outcome	Cancer sites	References
<i>Trp53</i> null × <i>Thbs1</i> null mice	Decreased survival; decreased incidence of osteosarcomas	Various	163
<i>MMTV-THBS1</i> × <i>MMTV-neu</i> mice	Delays and prevents tumors	Mammary gland	164
<i>K14-THBS1</i> mice: over-expression in skin: chemical carcinogenesis	delays squamous carcinogenesis but not malignant conversion	Skin	66
<i>UPII-SV40T</i> transgenic mice	Castration delays bladder carcinoma and increases TSP1	Bladder	165
polyomavirus middle Tag × <i>Thbs1</i> null mice	Larger primary tumors but fewer lung metastases	Mammary gland	125
<i>Thbs1</i> null mice	Antitumor effects of low- dose continuous cyclophosphamide lost in <i>Thbs1</i> -null	Syngeneic LL/2 tumors s.c.	166
<i>Thbs2</i> null mice: chemical carcinogenesis	Accelerated and increased tumor formation	skin	65
Human pathological studies	TSP1 or 2 decrease with progression	Colon carcinoma, penile squamous cell carcinoma, cervical carcinoma, leiomyosarcoma, melanoma, prostate cancer, neuroblastoma, breast ductal carcinoma in situ	58, 59, 63, 68, 167–174
	TSP1 or TSP2 increase with progression	non-Hogkin's lymphoma, colorectal liver metastases, vulvar squamous cell carcinoma, hepatocellular carcinoma	61, 175–177
	TSP1 and TSP2 not correlated with progression	stage III ovarian carcinoma, breast cancers	178, 179
	TSP1 expression attributed to tumor is actually from stroma	Melanoma, breast ductal carcinoma in situ, Pancreatic ductal carcinoma	62–64

* This table is meant to be an illustrative but not comprehensive listing of the observed relationships between thrombospondin and cancer progression.

MMTV, mouse mammary tumor virus; UPII, uroplakin II promoter; K14, Keratin 14 promoter

Table 2**Hypertensive and prothrombotic activities of angiogenesis inhibitors**

Drug	Side-effect (incidence)	Study design	Reference
Bevacizumab	Hypertension (35%)	154 patients: 20% new onset and 80% an exacerbation of pre-existing hypertension	1
Bevacizumab	Hypertension (15%)	Phase II trial in unresectable hepatocellular carcinoma	180
	Thrombosis (6%).		
Bevacizumab	Venous thromboembolism (11.9%)	Meta-analysis of 7956 patients from 15 randomized controlled trials	181
Bevacizumab	Hypertension (23–34%)	Review of clinical data for several cancers	182
Sorafenib	Hypertension (11–75%)		
Sunitinib	Hypertension (19%)		
Sorafenib	Hypertension (23.4%)	Meta-analysis of 4599 patients with renal cell carcinoma	2
Sorafenib	Hypertension and proteinuria (7 patients)	Noted to resemble preeclampsia	183
Sunitinib			
Cedifanib	Acute hypertension in rats	NO signaling proposed to be responsible	6
Telatinib	Hypertension and Proteinuria (18 patients)	Phase I trial: noted decreased response to nitroglycerin	184
Various inhibitors	Hemostasis and thrombosis	Review of clinical study and trial data	3
Various inhibitor	Hypertension and proteinuria	Editorial proposes key role for NO depletion in renal vasculature	120

NO, nitric oxide