Nitric Oxide and Its Gatekeeper Thrombospondin-1 in Tumor Angiogenesis

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Abstract
Nitric oxide (NO) plays a central role in angiogenesis as a mediator of signaling by vascular endothelial growth factor and other angiogenic factors. Low concentrations of NO produced in response to angiogenic factors stimulate angiogenesis, whereas higher concentrations typical of inflammatory responses inhibit angiogenesis. The proangiogenic activity of NO is mediated by activation of soluble guanylyl cyclase, leading to cyclic guanosine 3',5'-monophosphate accumulation and activation of its target kinases and ion channels. The four angiogenesis inhibitors currently approved for clinical use target components of the signaling cascade upstream of NO. New research has identified components downstream of NO as the primary target of the endogenous angiogenesis inhibitor thrombospondin-1 and has shown that circulating levels of thrombospondin-1 are sufficient to limit angiogenic responses by antagonizing NO signaling. This provides new insights into the significance of the widespread loss of thrombospondin-1 expression during malignant progression. Although clinical trials suggest that blocking NO signaling can inhibit tumor angiogenesis, this approach also inactivates inhibitory signaling from thrombospondin-1. We discuss the implications of the balance between these pathways for applying thrombospondin-1 mimetics and redox modifiers as cancer therapeutics.

Over the past decade, new cancer treatment and prevention strategies have been developed, employing agents that target the tumor vasculature. Rapidly growing tumors outpace the capacity of the surrounding microcirculation to provide for its metabolic needs (1–3). This results in recruitment of new vascular growth from an existing system of microvessels (angiogenesis) as well as creation of new vessels by recruitment of circulating endothelial precursors (4–8). Under normal conditions, angiogenesis is controlled through a balance of stimulating and inhibiting factors (9–11), resulting in selective and self-limited angiogenesis during normal wound healing (12–14). In contrast, uncontrolled angiogenesis is a hallmark of most cancers (15–17). One of the primary stimulators of angiogenesis in healthy and cancerous tissues is the bioactive gas, nitric oxide (NO; refs. 18–20), formed by NO synthase (NOS) using the substrate L-arginine.

NO signaling participates in many aspects of cancer and has both protumor and antitumor activities (21–23). For angiogenesis, a variety of studies have shown that NO can promote or inhibit neovascular formation (20, 24, 25). This dichotomy can be explained in part by the concentration and temporal dependencies of NO signaling pathways in endothelial cells. Low NO concentrations (<1-10 nmol/L) lead to cyclic guanosine 3',5'-monophosphate (cGMP)/extracellular signal-regulated kinase–dependent increases in endothelial cell proliferation and migration (26, 27). In contrast, NO concentrations >300 nmol/L, such as produced by activated macrophages, increased Ser15 phosphorylation on p53 and phosphorylation of MKP-1 (Fig. 1), causing cytostasis and at higher concentrations cell death (26, 27). These observations provide a mechanistic basis for the known biphasic effects of NO on endothelial cell proliferation, migration, and survival.

Mammalian cells express three isoforms of NOS that are regulated by different molecular mechanisms (28). These isoforms differ in their quantitative and temporal contributions to NO profiles associated with proangiogenic or antiangiogenic processes. Under physiologic conditions, endothelial NOS (eNOS) generates short bursts of NO (10-30 nmol/L), causing relaxation of smooth muscle cells (29). Phosphorylation of eNOS at Ser1179 leads to sustained low fluxes of NO (estimated to be 1-10 nmol/L; ref. 27) that stimulate proliferation of endothelial cells (24). Higher concentrations of NO produced by inducible NOS (iNOS) expressed in macrophages, when sustained for prolonged times, induce phosphorylation of p53 and induce MKP-1 and result in endothelial cell growth arrest (27). Such high levels of NO can be achieved in the microenvironment of human tissue under inflammatory conditions associated with diseases such as hepatitis and ulcerative colitis (30). In leukemia and other tumors, these higher concentrations of NO are required for antitumor immune responses. Thus, levels of NO >300 nmol/L produced by iNOS have antitumor and antiangiogenic properties. However, it should be noted that iNOS can generate a wide range of NO fluxes depending upon the stimuli and biological
production as well as VEGF-mediated phosphorylation of 17-(allylamino)-17-demethoxygeldanamycin decrease VEGF release. The antitumor agents geldanamycin and sorafenib (39), which decreases eNOS phosphorylation and eNOS/sGC/cGMP cascade.

However, several additional antiangiogenic agents under development regulate eNOS activity or other sites in the signaling cascade. Recent Advances in Redox Regulation by TSP1

TSP1 is a potent endogenous inhibitor of both physiologic and pathologic angiogenesis (42–44). TSP1 is a large trimeric glycoprotein that interacts with extracellular matrix components and with several cell surface receptors (43, 46). Normally found at low concentrations in the circulation and soft tissues, TSP1 expression increases significantly following wounding, and its expression is altered in a number of pathologic diseases (47). In cancer, TSP1 expression generally decreases with malignant progression, resulting from regulation of its expression by a number of oncogene and tumor suppressor gene products (42, 44), although stromal TSP1 expression in some cases masks this decrease. Experimental tumors with decreased TSP1 expression show significantly increased growth and metastasis (42). In contrast TSP1-overexpressing tumors typically grow slower, exhibit less angiogenesis, and have fewer metastases. Recently, we reported that the potent antiangiogenic agent thrombospondin-1 (TSP1) targets the downstream sites 3 and 4.

The NO/cGMP pathway is a convergence point for angiogenesis inhibitor signaling. Redox regulation of angiogenesis can be conceptualized in terms of a four-site model to distinguish the modes of action of angiogenesis inhibitors that act on upstream versus downstream sites in the signaling cascade. Site 1, eNOS is activated to synthesize NO by phosphorylation (P-eNOS) via the kinase Akt complexed with the chaperone heat shock protein 90 (Hsp90). Akt activity is stimulated by VEGF binding to its tyrosine kinase receptor VEGFR2 and by other proangiogenic growth factors, such as insulin, estrogen, and angiopoietin-1. All currently Food and Drug Administration–approved angiogenesis inhibitors act to sequester VEGF (Avastin and Lucentis) or inhibit the kinase activity of VEGFR2 and related kinases (Sunitinib and Sorafenib). Dephosphorylation of eNOS by the phosphatase PP2A is stimulated by the angiogenesis inhibitor endostatin.

Site 2, levels of NO are regulated by reaction with reactive oxygen species (ROS), including O2−, which is generated by the ansamycins geldanamycin and 17-(allylamino)-17-demethoxygeldanamycin (17-AAG). Site 3, low nanomolar levels of NO activate the soluble isofom of guanylyl cyclase (sGC) to synthesize cGMP. Binding of TSP1 to CD36 or CD47 is sufficient to inhibit cGMP synthesis, and CD47 is necessary for inhibition through both receptors. The drug ABT-510, currently in phase II trials, also targets CD36. Site 4, cGMP activates targets, including cGMP-dependent protein kinases and ion channels, to stimulate angiogenesis. Downstream phosphorylation and activation of the mitogen-activated protein kinase ERK is stimulated by cGMP but inhibited at high NO levels via induction of the mitogen-activated protein kinase phosphatase MKP1. TSP1 also inhibits site 4 signaling downstream of cGMP via an undefined mechanism.
through TSP1 interactions with its receptors CD36 and CD47 (26, 49). More importantly, in the presence of physiologic levels of NO, vascular cells become hypersensitive to the inhibitory effects of TSP1. Under these conditions, concentrations of TSP1 a thousand-fold less than normally effective completely block proangiogenic responses in vascular cells. These results suggest that low doses of NO donors could synergize with antiangiogenic therapies using TSP1 or drugs targeting its CD36 receptor (50).

The dramatic enhancement of the potency of TSP1 as an angiogenesis inhibitor in the presence of NO suggests that a major role of TSP1 is to antagonize the NO/cGMP pathway. Targeting downstream from eNOS may confer advantages for blocking angiogenesis in that endothelial cell signaling due to NO produced by other cells (i.e., iNOS from leukocytes) can also be blocked by TSP1. Furthermore, unlike Avastin and Lucentis, TSP1 can inhibit downstream signals resulting from angiogenic factors other than VEGF.

**Clinical-Translational Implications**

Based on these findings, two approaches have been considered to control cancer by manipulating NO. Inhibiting eNOS could limit the positive effects of low NO concentrations on tumor survival and angiogenesis. One difficulty in applying this strategy stems from the systemic side effects obtained with chronic administration of agents that block NO production (51, 52). Conversely, elevating NO to toxic levels could enhance cytotoxic therapies.

Depletion of the NOS substrate arginine has been applied in several cancer clinical trials. One approach used bacterial arginine deiminase to deplete circulating arginine. Phase I and II trials were completed for patients with metastatic melanoma and hepatocellular carcinomas (53, 54). In patients with unresectable hepatocellular carcinoma, arginine depletion was well tolerated: 16 of 19 treated patients showed a response, and mean survival was 410 days. In patients with metastatic melanoma, the therapy was also well tolerated, with 25% showing a response. In a related approach, arginine was depleted in hepatocellular carcinoma patients by inducing release of hepatic arginine (55). Five of seven patients treated achieved a depletion of arginine and showed a decrease in primary and metastatic tumor lesions. The NOS inhibitors l-NNAME and N\textsubscript{C}-monomethyl-L-arginine have been used in clinical trials in conjunction with interleukin-2 to ablate its hypotensive effects, but the NOS inhibitors were not separately evaluated in these trials for antitumor activities (56).

Increasing NO has also been examined in cancer clinical trials. In a randomized double blind trial of 31 patients with oral squamous carcinomas, administration of isosorbide mononitrite (20 mg bid) had no effect on cellular proliferation assessed by Ki67 immunohistology or on clinical status (57). NicOx nitroaspirin was approved for chemopreventive trials in patients at risk for colon cancer (58). A phase I trial of the chemopreventive effects of nitroaspirin in colon cancer at Stony Brook is currently ongoing (Rigas NCT00331786 and ref. 59).

Metronomic dosing, also known as antiangiogenic chemotherapy, involves the optimization of the effects of cytotoxic drugs by administering them continuously at low, nontoxic doses (44, 60). Metronomic dosing seems to provide a promising new approach because the targeted endothelial cells within the tumor bed are genetically stable and are therefore at a reduced risk of developing drug resistance, and low dosage produces significantly fewer side effects (44). Moreover, a recent report has shown that TSP1 secreted from the tumor microenvironment mediated the antiangiogenic and tumor suppressive effects of low-dose cyclophosphamide (60).

These observations have prompted the development of drugs that mimic an antiangiogenic domain of TSP1. A small-molecule angiogenesis inhibitor based on a CD36-binding peptide sequence from TSP1 (ABT-510) has completed phase I trials (61, 62) and is currently in phase II trials (50). Synergism of ABT-510 with metronomic chemotherapy (63) prompted combination phase I trials with 5-fluorouracil and leucovorin (64) or with gemcitabine and cisplatin (65). Based on our data that ligating the TSP1 receptor CD36 is sufficient to inhibit NO signaling, we predict that ABT-510 should share this activity, and that low-dose NO may potentiate its activity.

Our model predicts that strategies to target both sides of the angiogenic balance may be more efficacious. Exposure to low dose NO sensitizes vascular cells to TSP1. Therefore, concurrent treatment of tumors with low-dose NO and TSP1-based drugs should significantly increase the antiangiogenic potency of these drugs. Synergistic antitumor activity has also been found in combining radiation with TSP1 (66, 67). As does low-level NO treatment, radiation therapy alters the redox environment of the tumor. Thus, it is not surprising then that TSP1 is complimentary with radiotherapy of cancer.

**References**


