Endogenous Inhibitors of Angiogenesis

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Abstract

Angiogenesis, the formation of new blood vessels, is required for many pathologic processes, including invasive tumor growth as well as physiologic organ/tissue maintenance. Angiogenesis during development and adulthood is likely regulated by a balance between endogenous proangiogenic and antiangiogenic factors. It is speculated that tumor growth requires disruption of such balance; thus, the angiogenic switch must be turned "on" for cancer progression. If the angiogenic switch needs to be turned on to facilitate the tumor growth, the question remains as to what the physiologic status of this switch is in the adult human body; is it "off," with inhibitors outweighing the stimulators, or maintained at a fine "balance," keeping the proangiogenic properties of many factors at a delicate "activity" balance with endogenous inhibitors of angiogenesis. The physiologic status of this balance is important to understand as it might determine an individual's predisposition to switch on the turn on during pathologic events dependent on angiogenesis. Conceivably, if the physiologic angiogenesis balance in human population exists somewhere between off and even balance, an individual's capacity and rate to turn the switch on might reflect their normal physiologic angiogenic status. In this regard, although extensive knowledge has been gained in our understanding of endogenous growth factors that stimulate angiogenesis, the activities associated with endogenous inhibitors are poorly understood. In this review, we will present an overview of the knowledge gained in studies related to the identification and characterization of 27 different endogenous inhibitors of angiogenesis. (Cancer Res 2005; 65(10): 3967-79)

Introduction

Angiogenesis, the formation of new capillaries, is among the key events in various tissue destructive pathologic processes, such as tumor growth, metastasis, arthritis, etc., as well as in physiologic processes, like organ growth and development, wound healing, and reproduction (1). A hypothesis that tumor growth is angiogenesis dependent was first proposed by Folkman (2). The expansion of tumors is strictly dependent on the neangiogenesis, and the inhibition of vascular supply to tumors can suppress their growth (3). Solid tumors cannot grow beyond 2 to 3 mm in diameter without being able to recruit their own blood supply. Angiogenesis disrupts with this process. It also inhibits the formation of new blood vessels in Matrigel plug assay in mice (10). Arresten inhibits endothelial cell tube formation (10). Normally, when mouse aortic endothelial cells are cultured on Matrigel (a solid gel of mouse basement membrane proteins), they rapidly align and form hollow tube-like structures (11), but arresten disrupts with this process. It also inhibits the formation of new blood vessels in Matrigel plug assay in mice (10). Arresten inhibits endothelial cell proliferation and migration (10). Arresten does not have a significant effect on the proliferation of several cancer cell lines even at very high doses. Arresten affects metastasis leading to significant reduction of pulmonary nodules in arresten-treated factor, and hypoxic conditions that activate hypoxia-inducible factor-1, which itself can up-regulate angiogenic proteins, as well as angiogenic oncogenes, such as Ras, and tumor suppressors, such as p53. Endogenous inhibitors of angiogenesis include various antiangiogenic peptides, hormone metabolites, and apoptosis modulators (Fig. 2; for reviews, see refs. 1, 4). To understand how systemic angiogenesis balance in the body is regulated by endogenous inhibitors of angiogenesis requires an understanding of how many such molecules exist and their role in inhibiting angiogenesis. This review will highlight the important features of each of these molecules and address their role in the regulation of angiogenesis balance.

Vascular basement membrane components can modulate endothelial cell behavior in addition to providing structural and functional support (5). A series of endogenous antiangiogenic factors have been described, of which many are fragments of naturally occurring extracellular matrix (ECM) and basement membrane proteins (6). In this review, we divide the endogenous inhibitors of angiogenesis into two major classes, matrix-derived inhibitors and non-matrix-derived inhibitors (Fig. 2).

The reader will notice that most studies dealing with endogenous inhibitors of angiogenesis address therapeutic impact of these agents to control cancer progression when given at pharmacologic doses. Nevertheless, interesting studies are emerging, which also highlight the role of these inhibitors in controlling cancer at physiologic concentrations.

Matrix-Derived Inhibitors of Angiogenesis

Arresten. The main component of vascular basement membranes is type IV collagen, forming a mesh-like structure with other macromolecules, such as laminin, heparan sulfate proteoglycans, fibronectin, and entactin (7). Type IV collagen is composed of six distinct gene products (i.e., α1-α6; for a review, see ref. 8). It has been observed earlier that α1 and α2 type IV collagen chains isolated from the Engelbreth-Holm-Swarm sarcoma tumor are inhibitory to capillary endothelial cell proliferation (9).

Arresten is a recently identified endogenous inhibitor of angiogenesis. It is a 26-kDa molecule derived from the non-collagenous (NC1) domain of the α1 chain of type IV collagen (10). In endothelial cell proliferation assays, a dose-dependent inhibition of basic FGF (bFGF)–stimulated endothelial cells proliferation is detected with an ED50 value of as low as 10 nmol/L. Arresten selectively inhibits endothelial cell tube formation (10). Normally, when mouse aortic endothelial cells are cultured on Matrigel (a solid gel of mouse basement membrane proteins), they rapidly align and form hollow tube-like structures (11), but arresten disrupts with this process. It also inhibits the formation of new blood vessels in Matrigel plug assay in mice (10). Arresten inhibits endothelial cell proliferation and migration (10). Arresten does not have a significant effect on the proliferation of several cancer cell lines even at very high doses. Arresten affects metastasis leading to significant reduction of pulmonary nodules in arresten-treated...
mice and inhibition of large and small renal cell carcinoma tumor growth (10).

The existing evidence suggests that arresten might function via a₁b₁ integrin and block the binding of a₁b₁ integrin to the type I collagen (10). a₁b₁ Integrin is a collagen receptor, but it also binds to other basement membrane constituents. In this regard, a₁ integrin neutralizing antibodies can suppress angiogenesis associated with tumor growth (12). The b₁ integrin is also involved in angiogenesis (13). Ablation or blocking of the interactions with integrin a₁b₁ inhibits angiogenesis (12, 14), indicating that integrin a₁b₁ acts as a proangiogenic factor. Among the collagen integrins, the a₁b₁ integrin activates the Ras-Shc-mitogen-activated protein kinase (MAPK) pathway, thus promoting cell proliferation (15). In addition, in the a₁ integrin–deficient mice, tumors are less vascularized compared with wild-type mice (14, 16). Arresten might also function via binding to heparan sulfate proteoglycan (10) and previous studies have shown that heparan sulfate proteoglycan binds to the a₁ NC1 domain of type IV collagen (17).

Canstatin. Canstatin is a 24-kDa fragment of the a₂ chain of type IV collagen. Recombinant canstatin significantly inhibits endothelial cell migration and tube formation in a dose-dependent manner (18). Canstatin inhibits serum-stimulated human endothelial cell proliferation and induces apoptosis with no inhibitory effect on proliferation or apoptosis of nonendothelial cells. The inhibition of endothelial proliferation was not associated with a change in extracellular signal-regulated kinase (ERK) activation, as canstatin did not alter the early phosphorylation of ERK after stimulation with serum and/or mitogens. This indicates that canstatin does not primarily work by inhibiting proximal events activated by VEGF or bFGF receptors. It is possible that canstatin acts by predisposing cells to apoptosis. Canstatin also suppresses growth of tumors in human xenograft mouse models, with histology revealing decreased CD31-positive vasculature (18). It inhibits the phosphorylation of Akt, focal adhesion kinase (FAK), mammalian target of rapamycin (mTOR), eukaryotic initiation factor 4E-binding protein-1 (4E-BP1), and ribosomal S6 kinase in cultured human umbilical vein endothelial cells (HUVEC). It also induces Fas ligand (FasL) expression, activates procaspase-8 and -9 cleavage, reduces mitochondrial membrane potential, and increases cell death. Thus, canstatin-induced apoptosis seems to be associated with phosphatidylinositol 3-kinase (PI3K)/Akt inhibition and is dependent on signaling events transduced through membrane death receptors (19). A functional receptor for canstatin is not yet identified, but canstatin likely functions via cell surface integrins.

In addition to the NC1 domains of collagen IV a₁, a₂, and a₃ (see below) chains, the a₆ chain possesses antiangiogenic activity and
inhibits tumor growth. Soluble NC1 domain of the α6 chain regulates endothelial cell adhesion and migration (20).

**Endorepellin.** Perlecan is basement membrane heparan sulfate proteoglycan that plays key roles in vascular growth (21). The COOH-terminal end of perlecan, called endorepellin or perlecan domain V, potently inhibits several aspects of angiogenesis: endothelial cell migration, collagen-induced endothelial tube morphogenesis, and blood vessel growth in the chicken chorioallantoic membrane assay and in mouse Matrigel plug assays. Endorepellin is active at nanomolar concentrations in the *in vitro* assays and blocks endothelial cell adhesion to fibronectin and type I collagen without directly binding to these matrix proteins (22). Endorepellin binds to endothelial cells as well as to squamous cell carcinoma cells and breast carcinoma cells via high-affinity receptors. Interestingly, endorepellin binds endostatin, another matrix-derived inhibitor of angiogenesis, and counteracts its antiangiogenic effects (22). The exact mechanism of endorepellin is not yet known. In this regard, β1 integrin and α-dystroglycan have been shown to interact with the COOH-terminal domain of perlecan (a region containing endorepellin; refs. 23–25).

**Endostatin.** Endostatin is an endogenous collagen XVIII–derived angiogenesis inhibitor identified and purified from murine hemangioendothelioma cell line (26) and later characterized in mice (27). It corresponds to a 20-kDa fragment derived from the COOH-terminal NC1 domain of type XVIII collagen (26, 28, 29). Recombinant endostatin efficiently blocks angiogenesis and suppresses primary tumor growth and metastasis in experimental animal models without any apparent side effects, toxicity, or development of drug resistance (26, 30, 31).

New insights into the molecular mechanisms associated with endostatin for inhibition of tumor growth are emerging. Recent studies have reported that endostatin interferes with FGF-2-induced signal transduction, blocking endothelial cell motility (32), inducing apoptosis (33), causing G1 arrest of endothelial cells through inhibition of cyclin D1 (34), blocking VEGF-mediated signaling via direct interaction with the VEGF-R2/KDR/Flik-1 receptor tyrosine kinase in HUVECs (35), and blocking tumor necrosis factor–induced activation of c-Jun NH2-terminal kinase and c-Jun NH2-terminal kinase–dependent proangiogenic gene expression (36). Endostatin rapidly down-regulates many genes in growing endothelial cells, including immediate-early response genes, cell cycle–related genes, and genes regulating apoptosis inhibitors, MAPKs, FAKs, and G-protein-coupled receptors mediating endothelial cell growth, mitogenic factors, adhesion molecules, and cell structure components (37). Recently, it is shown that endostatin down-regulates many signaling pathways in human microvascular endothelium associated with proangiogenic activity and at the same time up-regulating many antiangiogenic genes. Endostatin also affects signaling events that are not associated with angiogenesis, demonstrating the importance of interpathway communications in an intricate signaling network (38).

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**Figure 2.** The endogenous angiogenesis inhibitors.
Rehn et al. showed that recombinant “immobilized” human endostatin interacts with α5 and αv integrins on the surface of human endothelial cells. Furthermore, this endostatin-integrin interaction is of functional significance in vitro, as immobilized endostatin promotes integrin-dependent endothelial cell functions (39). Sudhakar et al. have further studied the receptors for soluble endostatin and the downstream signaling events. Endostatin binds to the α5β1 integrin and inhibits the migration of endothelial cells by blocking signaling pathways via Ras and Raf and further downstream via ERK1 or p38 (Fig. 3; ref. 40). In the endothelial cells, endostatin induces rapid clustering of α5β1 integrin associated with actin stress fibers and causes colocalization with the membrane anchor protein caveolin-1, which couples integrins to cytoplasmic signaling cascades (41). In these experiments, it was shown that endostatin binds to both α5β1 and caveolin-1 and that endostatin treatment induces phosphatase-dependent activation of caveolin-associated Src family kinases (42). The disassembly of actin stress fibers/local adhesions by endostatin effects cell-matrix interaction and cell motility via activation of Src and in a tyrosyl phosphatase–dependent manner (42). On the other hand, Eriksson et al. conclude that endostatin inhibits chemotaxis, without affecting intracellular pathways known to regulate endothelial cell migration and proliferation/survival, because they did not find any effect of endostatin on phospholipase C-γ, Akt/protein kinase B, p44/42 MAPK, p38 MAPK, and p21-activated kinase activity (43). Moreover, a recent study shows that endostatin action is dependent on expression of E-selectin on the endothelial cells, although a direct binding of endostatin to E-selectin was not observed (44). Therefore, more work needs to be done to sort out the exact mechanism of action associated with endostatin.

Endostatin binds to heparin (45) and with low affinity to all surface heparan sulfate proteoglycans that are involved in growth factor signaling (46–48). The antiangiogenic activity of endostatin seems to depend on the interactions with heparan sulfate proteoglycans possibly by an interaction between discontinuous sulfated domains in heparan sulfate proteoglycans and arginine clusters at the endostatin surface (49). Recent work has shown the identity of a specific arginine-rich sequence motif of human endostatin that interacts with endothelial cell surface β1 integrin and heparin and inhibits endothelial cell migration and tube formation. This Arg-Gly-Asp (RGD)–independent sequence is likely to be the motif responsible for the antiangiogenic activity of endostatin (50).

Endostatin inhibits the activation and activity of certain matrix metalloproteinases (MMP; i.e., MMP-2, -9, and -13 and MT1-MMP) and it binds directly to at least MMP-2 and -9 (51–53). However, endostatin does not inhibit all MMPs, because it had no effect on the activation of MMP-8 (53). In addition to MMPs, endostatin has...
been shown to interfere with the actions of other proteases, like the plasminogen activator system (54). Interestingly, certain MMPs can generate endostatin-containing peptides differing in molecular size (20-30 kDa) from human type XVIII collagen (55). These fragments inhibit the proliferation and migration of HUVECs in a similar fashion as native 20-kDa endostatin.1

Endostatin has been shown to significantly reduce invasion of not only endothelial cells but also tumor cells into reconstituted basement membrane, Matrigel (51). Although the main focus has been on the antiangiogenic activity of endostatin, it should also be noted that endostatin inhibits tumor growth not only by acting via endothelial cells but also by directly decreasing carcinoma cell migration. Furthermore, intravasation (the key step of carcinoma process leading to metastasis, where tumor cells invade through blood vessel walls into the bloodstream) of oral carcinoma cells is inhibited by endostatin (53).

Endostatin can exist in two forms: as a monomer or as a trimer that exerts different or even opposite effects (e.g., trimeric endostatin in the NCI domain is needed for endothelial cell migration, but monomeric endostatin inhibits the migratory activity; refs. 56, 57). In addition, it has been shown recently that endostatin can exist as a soluble globular form or as an insoluble form with abundant cross-β-sheets aggregating into amyloid deposits. Such different endostatin conformations have distinct effects on plasminogen activation. Insoluble endostatin stimulates plasminogen activation, whereas soluble endostatin has no effect on plasminogen activation (58).

The physiologic levels of circulating endostatin in the serum are ~40 to 100 ng/mL compared with the concentrations of endostatin (0.2-20 mg/mL) that are effective in the inhibition of tumor growth in various experiments. It has been shown that some of the antiangiogenic and antitumor effects of endostatin might be in fact represent pharmacologic effects at high doses and not necessarily related to the physiologic function of endostatin (59). In this regard, the physiologic levels of endostatin have no effect on the growth of fibrosarcomas and melanomas in collagen XVIII/ endostatin knockout mice (60).

Endostatin-like fragment from type XV collagen. Based on a homology search with endostatin, a 22-kDa fragment of collagen XV was found with 70% homology to endostatin. It inhibits the migration of endothelial cells but has no effect on proliferation. Systemic administration of endostatin-like fragment from type XV collagen (EFC-XV) suppresses the growth of tumors in a xenograft renal carcinoma model (61). Both NCI domains of collagen XV and XVIII contain a trimerization domain, a hinge region that is more sensitive to proteolysis in collagen XVIII than collagen type XV. Unlike endostatin, EFCXV does not bind to zinc or heparin. Both endostatin and EFC-XV inhibit FGF-2-induced or VEGF-induced angiogenesis associated with chicken chorioallantoic membrane angiogenesis, but there are striking differences depending on which cytokine is used and whether free EFC-XV or XV-NC1 domains are used (62). Endostatin and EFC-XV show a similar binding repertoire for ECM proteins. However, differences are detected using immunohistologic localization in vessel walls and basement membrane zones (62). Although it seems that the functions of endostatin and EFC-XV somewhat overlap, double knockout mice show no additional defects compared with the single knockout mice (63).

Anastellin—a fibronectin fragment. Incubation of soluble fibronectin with a small fibronectin-derived fragment, called anastellin, results in a polymeric form of fibronectin that is strongly antiangiostatic in tumor-bearing mice (64). Both anastellin and polymeric fibronectin reduce tumor growth in mice, and the tumors are less vascularized (65). Anastellin is unable to inhibit Matrigel plug angiogenesis in mice that lack plasma fibronectin, but it is fully active in mice that are null for vitronectin, which like fibronectin is a major endothelial cell adhesion protein. Interestingly, the activity of endostatin is impaired in both fibronectin-deficient and vitronectin-deficient mice. This suggests a shared mechanism of action for antiangiogenic factors derived from ECM and plasma proteins (66).

Fibulins. Proteolytic digestion of basement membrane preparations by elastases and cathepsins releases fragments, which possess antiangiogenic activity (67). Two such fragments were COOH-terminal fragments corresponding to fibulin 1D and the domain III of fibulin 5.2 Recently, fibulin 5 has been shown to antagonize VEGF signaling and inhibit endothelial angiogenic sprouting (68). Fibulin 5 also binds to extracellular superoxide dismutase (69). In contrast, another report recently shows that fibulin 5 promotes wound healing in vivo (70). Considering that wound healing is dependent on angiogenesis, this report opposes the notion that fibulin 5 is an inhibitor of angiogenesis. Future studies will hopefully shed more light on this opposing action of fibulin 5.

Thrombospondins. Thrombospondin-1 (TSP-1) was the first protein to be recognized as a naturally occurring inhibitor of angiogenesis (71). It is a large multifunctional ECM glycoprotein that regulates various biological events, like cell adhesion, angiogenesis, cell proliferation and survival, transforming growth factor-β (TGF-β) activation, and protease activation (ref. 63; for a review, see ref. 72). Some studies suggest that TSP-1 may possess dual activity (proangiogenic and antiangiogenic) depending on proteases that generate fragments of TSP-1 (73, 74). It has been shown to inhibit tumor growth and metastasis, thus making it a potent inhibitor of in vivo neovascularization and tumorigenesis. Overexpression of TSP-1 in mice suppresses wound healing and tumorigenesis, whereas the lack of functional TSP-1 results in increased vascularization of selected tissues (75–77). Expression of TSP-1 has been inversely correlated with malignant progression in breast and lung carcinomas and melanomas (78). To evaluate the importance of TSP-1 for the progression of naturally arising tumors in vivo, Lawler et al. have crossed TSP-1-deficient mice with p53-deficient mice. In the p53-null mice, the absence of TSP-1 decreased survival. They also determined more directly whether host TSP-1 inhibited tumor growth by implanting melanoma and testicular teratocarcinoma cells into the TSP-1-null mice. The tumors grew faster in the TSP-1-null background and exhibited an increase in vascular density, a decrease in the rate of tumor cell apoptosis, and an increase in the rate of tumor cell proliferation (79). The antiangiogenic activity of TSP-1 has been mapped to the type 1 repeats and within the NH₂-terminal portion of the molecule within the procollagen-like domain. TSP-1 and peptides from the type 1 repeat region (tryptophan-rich, heparin-binding sequences and TGF-β1 activation sequences) were evaluated in two models of retinal angiogenesis. TSP-1 inhibited angiogenesis in both experimental models, but peptides from the native TSP-1 sequence

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1 Heljasvaara et al., unpublished observations.

2 Xie and Kalluri, unpublished data.
containing both the tryptophan-rich repeat and the TGF-β1 activation sequence or containing only the tryptophan-rich, heparin-binding sequence had distinct efficiencies in the two models. These results suggest that the type 1 repeats of TSP-1 contain two subdomains that might independently influence the process of neovascularization (80). The existence of two subdomains also explains how TSP-1 may block FGF-2 and VEGF angiogenic signals by two independent pathways (81). TSP-1 is able to distinguish pathologic neovascularization from preexisting vasculature due to the dependence of proliferating endothelial cells on Fas/FasL-mediated apoptosis. TSP-1 up-regulates FasL expression on endothelial cells. Expression for the receptor of FasL, was low on quiescent endothelial cells but greatly enhanced by inducers of angiogenesis, thereby specifically sensitizing the stimulated cells to apoptosis by inhibitor-generated FasL (82).

TSP-2 also shows antiangiogenic activity. Injection of TSP-2-transfected squamous cell carcinoma cells into the dermis of nude mice resulted in inhibition of tumor growth that was even stronger than the inhibition observed with TSP-1-transfected cells. The combined overexpression of TSP-1 and TSP-2 completely prevented tumor formation. Extensive areas of necrosis were observed in TSP-2-expressing tumors, and both the density and the size of tumor vessels were significantly reduced (83). Furthermore, tumor angiogenesis was significantly enhanced in TSP-2-deficient mice. Although TSP-2 deficiency did not affect tumor differentiation or proliferation, tumor cell apoptosis was significantly reduced (84). The antiangiogenic role of TSP-2 was further confirmed with an implant system that continuously produces TSP-2. Fibroblasts, which overexpressed TSP-2 and transplanted into nude mice resulting in increased levels of circulating TSP-2, inhibited tumor growth and angiogenesis of human squamous cell carcinomas, malignant melanomas, and Lewis lung carcinomas implanted at a distant site (85). It has been shown recently that the antiangiogenic region of TSP-2 lies approximately within the 80-kDa fragment of the NH2-terminal globular region (86). Daily injections of TSP-2 resulted in a significant inhibition of the growth of human squamous cell carcinomas in vivo and reduced tumor vascularization. Possible mechanisms for this antiangiogenic activity are inhibition of VEGF-induced endothelial cell migration, tube formation, and increased endothelial cell–specific apoptosis (86).

**Tumstatin.** Synthetic peptides derived from the NC1 domain of α3 chain of type IV collagen, residues 183 to 205, have been shown to inhibit the proliferation of melanoma and other epithelial tumor cell lines in vitro (87, 88) and bind to the CD47/α5β3 integrin complex (89). The entire 28-kDa fragment of α3 chain of NC1 domain of type IV collagen was named tumstatin (90, 91). In vivo overexpression of tumstatin domains by tumor cells inhibits their invasive properties in a mouse melanoma model (92). Tumstatin inhibits formation of new blood vessels in Matrigel plug assays and suppresses tumor growth of human renal cell carcinoma and prostate carcinoma in mouse xenograft models. This is associated with in vivo endothelial cell–specific apoptosis. The antiangiogenic activity is localized to a distinct region of the tumstatin molecule that is separate from the region responsible for the antitumor cell activity (90). Tumstatin has two binding sites for α5β3 integrin, one in the NH2-terminal end of the molecule (containing amino acids 54-132) that is associated with the antiangiogenic properties and the other in the COOH-terminal end (containing amino acids 185-203) that is associated with the antitumor cell activity (89, 91, 93). The tumstatin fragment containing amino acids 54 to 132 binds to both endothelial cells and melanoma cells but only inhibits proliferation of endothelial cells, with no effect on tumor cell proliferation. On the other hand, the fragment of tumstatin containing amino acids 185 to 203 binds also to both endothelial cells and melanoma cells but only inhibits the proliferation of melanoma cells. The presence of cyclic RGD peptides do not compete for the α5β3 integrin–mediated activity of tumstatin, although this peptide shows significant inhibition of endothelial cell binding to vitronectin and fibronectin. These two distinct binding sites on tumstatin suggest unique α5β3 integrin–mediated mechanisms governing the two distinct antitumor activities (91). A third proximal site, RGD site, capable of binding to αβ3 integrin, outside the NC1 domain sequence of tumstatin, has also been proposed recently (94). How these different α5β3 integrin sites regulate the antiangiogenic activity of tumstatin is not understood completely.

The antiangiogenic domain of tumstatin within the 54– to 132–amino acid region was named Tum-5. Recombinant Tum-5 inhibits endothelial cell tube formation on Matrigel and induces G1 endothelial cell cycle arrest. Tum-5 possesses antiangiogenic activity and inhibits human prostate cancer growth in association with a decrease in CD31-positive vasculature in nude mice (95). More specifically, the antiangiogenic activity of tumstatin is localized to a 25–amino acid region within the 69– to 98–amino acid area as shown with overlapping synthetic peptides. Both of these peptides are antiangiogenic and function via α5β3 integrin in a similar fashion as the full-length tumstatin (96).

The antiangiogenic and proapoptotic activity of tumstatin is specific for endothelial cells. Maeshima et al. show that tumstatin functions as an endothelial cell–specific inhibitor of protein synthesis. Through an interaction with α5β3 integrin, tumstatin inhibits activation of FAK, PI3K, protein kinase B/Akt, and mTOR and prevents the dissociation of eukaryotic initiation factor 4E protein from 4E-BP1 leading to the inhibition of Cap-dependent protein synthesis. Furthermore, these results establish a role for integrins in mediating cell-specific inhibition of Cap-dependent protein synthesis and suggest a potential mechanism for the selective effects of tumstatin on endothelial cells. Consequently, tumstatin causes endothelial cell apoptosis by inhibiting signaling pathways involved in protein synthesis via interaction with α5β3 integrin (97). It is known that endothelial cells adhere to immobilized VEGF mediated by α5β3, αβ3, as well as other αβ integrins but not by VEGF receptors (VEGFR). This adhesion almost totally abolishes endothelial cell apoptosis through the interaction with integrins. Tumstatin can inhibit the adhesion between endothelial cells and immobilized VEGF via the interaction with α5β3 integrin; thus, it induces apoptosis (98).

Although tumstatin and endostatin are both antiangiogenic molecules derived from the NC1 domains of basement membrane collagens, they share only 14% amino acid homology and their activities seem to be mediated via distinct signaling pathways. Human tumstatin prevents angiogenesis via inhibition of endothelial cell proliferation and promotion of apoptosis with no effect on migration, whereas human endostatin prevents endothelial cell migration with no effect on VEGF-induced proliferation. The reason for these distinct functions of endostatin and tumstatin can be explained partly by different integrin-binding capabilities and induction of different signaling pathways. The activity of tumstatin is mediated via binding to α5β3 integrin and inhibition of protein synthesis via FAK/P3K/Akt/mTOR/4E-BP1 pathway, whereas the activity of endostatin is mediated by αβ3 integrin and inhibition of FAK/c-Raf/MAPK-ERK kinase-1/2/p38/ERK1/2 MAPK pathway, with no effect on Cap-dependent protein synthesis (40).
Does the physiologic level of endogenous circulating tumstatin (~300-360 ng/mL in mice) have an effect on angiogenesis and tumor growth? Tumors in collagen IV α3 chain–deficient mice (that are concomitantly deficient in tumstatin as well) grow faster than in wild-type mice. Furthermore, administration of exogenous tumstatin (missing physiologic levels) decreases the growth back to the wild-type level (99). However, physiologic angiogenesis associated with tissue repair was not affected in these tumstatin-deficient mice. As mentioned before, the antiangiogenic activity of tumstatin is mediated by integrin αvβ3 (40, 91, 96). This was further confirmed by showing that tumstatin has no effect on the proliferation of mouse lung endothelial cells deficient in integrin β3 or on neovascularization of Matrigel plugs in the integrin β3–null mice (99). A clinical correlation study between levels of tumstatin and tumor progression of human bronchopulmonary carcinoma was done recently, which highlights the potential significance of tumstatin as an endogenous angiogenesis inhibitor (100). A clear correlation between tumstatin expression and mildly developed vascular networks was observed (100).

MMPs are capable of degrading type IV collagen and liberating fragments containing tumstatin (101). MMP-9 is the most effective in cleaving tumstatin-containing fragments from type IV collagen, but MMP-2, -3, and -13 also can release tumstatin. MMP-9-deficient mice have decreased levels of tumstatin in their blood, and the tumors in MMP-9-null mice grow faster than the tumors in wild-type mice (99).

Recent studies also show that tumstatin peptide can prevent glomerular hypertrophy in the early stages of diabetic nephropathy (102). Increase in the renal expression of VEGF, Flk-1, and Ang-2 was inhibited by tumstatin in mice with diabetic nephropathy (102).

**Non-Matrix-Derived Inhibitors of Angiogenesis**

**Angiostatin.** Cleavage of plasminogen (contains five kringles) by proteases results in the formation of 38- to 45-kDa antiangiogenic peptides that contain homologous triple-disulfide bridged kringle domains: kringle-1 to -4 or kringle-1 to -3. They are collectively called angiostatin (103–105). Subsequent studies have also shown that plasminogen kringle-5 by itself also exhibits antiangiogenesis activity (106). Angiostatin is a cryptic fragment of plasminogen that possesses antiangiogenic properties, a property not shared by the parent molecule (plasminogen). Angiostatin was originally purified from serum and urine of mice bearing s.c. Lewis lung carcinoma, where the growth of metastases was inhibited by tumor-generated angiostatin (107). Several members of the human MMP family, including matrixin (MMP-7) and gelatinase A and B (MMP-9 and -2), metalloelastase (MMP-12), and stromelysin-1 (MMP-3), hydrolyze human plasminogen to generate angiostatin fragments (104, 108–110) after plasminogen is converted to plasmin by plasminogen activator, which is followed by reduction by phosphoglycerate kinase (111). Angiotatin3ee inhibits endothelial cell proliferation and migration (107). It is suggested that the different kringle domains may contribute to the overall antiangiogenic function of angiostatin by their distinct antiangiocrine activities (e.g., kringle-4, the potent fragment in inhibiting endothelial cell migration, which has only marginal antiproliferative activity). In contrast, kringle-1 to -3, which is equivalent to angiostatin in inhibiting endothelial cell proliferation, manifests only a modest antiangiocrine effect (112).

There are several targets proposed for the action of angiostatin (for review, see ref. 113). It binds directly to the ATP synthase on the surface of endothelial cells, which might play a role in allowing the intracellular pH to drop, thus triggering apoptotic events in endothelial cells (114). Both angiostatin (which contains the RGD motif) and plasmin specifically bind to αvβ3 integrin, and angiostatin significantly inhibits plasmin-induced cell migration. This suggests that that binding of plasmin to αvβ3 integrin is required for its activity, and angiostatin may interfere with such activity (115, 116). Angiomotin is also a potential target for angiostatin, as it has been shown that cells that contained angiomotin are able to bind and internalize angiostatin, leading to an induction of FAK activity (117). In addition, Claesson-Welsh et al. found that treatment with angiostatin has no effect on growth factor–induced signal transduction but leads to a RGD-independent induction of FAK activity. They also show that angiostatin treatment of endothelial cells in the absence of growth factors results in increased apoptosis, whereas proliferation does not change. Angiostatin also inhibits migration and tube formation associated with proliferating endothelial cells (118).

**Cleaved antithrombin III and prothrombin kringle-2.** Circulating clotting factors in the blood seem to play an important role in angiogenesis. In addition to angiostatin (see above), TSPs (see above), and platelet factor-4 (PF-4; see below), the antiangiogenic forms of antithrombin III (119) and prothrombin kringle-2 (120) have been shown to posses antiangiogenic properties. Cleavage of the COOH-terminal loop of antithrombin induces a conformational change in the molecule, and the cleaved conformation has potent antiangiogenic and antitumor activity in mouse models. In addition, the latent form of intact antithrombin, which is similar in conformation to the cleaved molecule, also inhibits angiogenesis and tumor growth (119). In this regard, prothrombin kringle-2 domain also exhibits anti–endothelial cell proliferative activity (120).

**Chondromodulin-I.** Although cartilage contains many angiogenic factors during ossification, in adults it is an avascular tissue. The 25-kDa cartilage-specific NCI matrix protein chondromodulin-I is a strong inhibitor of angiogenesis (121). The expression pattern of the gene suggests a role for chondromodulin-I in the morphogenesis during embryonic development (122). The level of chondromodulin-I transcripts is substantially reduced in chondrosarcomas or in other benign cartilage tumors. Human chondrosarcoma cells in nude mice produce tumors with cartilaginous matrix. Local administration of recombinant human chondromodulin-I almost completely blocks vascular invasion and tumor growth in vivo. Furthermore, it inhibits the growth of colon adenocarcinoma in vivo, implying therapeutic potential for other solid tumors (123).

**Soluble Fms-like tyrosine kinase 1.** The soluble version of VEGFR-1 [soluble Fms-like tyrosine kinase 1 (sFlt-1)] was identified by Kendall et al. (124, 125). In addition to the full-length receptor, VEGFR-1 gene encodes for a small soluble form (sFlt-1) carrying only six Ig domain via an attenuate splicing (124, 126, 127). sFlt-1 has a strong affinity for VEGF and placental growth factor (124, 128, 129). In human, VEGF circulates at 75 pg/mL and sFlt-1 circulates at a concentration of 23 ng/mL (130). Therefore, VEGF can circulate in a bound state (bound to sFlt-1) as well as in free state depending on the amount of sFlt-1 in the circulation. In this regard, several reports have suggested that sFlt-1 can serve as an antitumor agent by inhibiting VEGF (130–132). The relevance for
sFlt-1 at the normal physiologic concentration in the regulation of cancer progression is not understood.

**Interferons.** IFNs, pleiotropic cytokines that regulate antiviral, antitumor, apoptotic, and cellular immune responses, were the first endogenous antiangiogenic regulators identified. IFNs inhibit angiogenesis induced by tumor cells in mice (133). IFN-α or IFN-β has biological activity against squamous cell carcinomas and inhibits angiogenesis in tumor-bearing nude mice. When tumor cells are treated in vitro with low-dose IFN-α, there is a significant drop in their secretion of interleukin (IL)-8, the major angiogenic factor produced by tumors (134). IFN-α reduces urokinase-type plasminogen activator and plasminogen activator inhibitor-1 activity (135) and significantly inhibits MMP-9 enzymatic activity and protein expression (136). IFN-α and IFN-β treatment also inhibits angiogenesis by down-regulation of bFGF expression (137–139), but other studies suggest that the action of IFN-α is not mediated by bFGF or VEGF (140–142).

**Interleukins.** ILs are a family of leukocyte-derived proteins with broad-ranging effects on multiple physiologic properties, including angiogenesis. ILs bearing a NH₂-terminal Glu-Leu-Arg (Erl) motif, such as IL-8, tend to display proangiogenic properties, whereas those lacking this motif have been found to inhibit angiogenesis (143). IL-1, mainly secretable IL-1β, is involved in inflammation, tumor growth, and metastasis. It is shown that IL-1β inhibits FGF-stimulated angiogenesis by an autocrine pathway (144). However, recent study found that the IL-1 receptor antagonist reduced inflammatory responses and inhibited tumor development in mice (145). IL-4 inhibits bFGF-induced angiogenesis (146). IL-12 and IL-18 are IFN-γ-inducing cytokines. IL-12 can inhibit angiogenesis through the downstream chemokines, such as IFN-inducible protein-10 and monokine induced by IFN-γ (147–149). Tumors in mice treated with IL-12 are significantly smaller and have extensive necrosis compared with untreated tumors (150). The combination of IL-12 and IL-2 mediated synergistic antitumor activity in preclinical tumor models (151). The IL-12 plasmid DNA gene transfer significantly prevented the growth and vascularization of highly angiogenic sarcoma and TS/A murine mammary carcinoma tumors in nude and/or syngeneic mice (152). IL-18 inhibits FGF-stimulated endothelial cell proliferation in vitro and suppresses the FGF-induced corneal neovascularization by systemic administration in mice (153). IL-18 can also inhibit embryonic angiogenesis.

**2-Methoxyestradiol.** 2-Methoxyestradiol (2-ME), an endogenous estradiol metabolite, is an inhibitor of angiogenesis, with direct effect on cancer cells (154–157). 2-ME is orally active molecule with low affinity for estrogen receptors (158). Its mechanism of action is attributed to its ability to bind to the colchicine binding site of tubulin and the inhibition of superoxide dismutase enzymatic activity (154, 159–161). Recently, another novel mechanism for 2-ME was proposed (162). 2-ME-mediated destabilization of microtubules was associated with a block in nuclear accumulation and activity of hypoxia-inducible factor-1α (oxygen and proteosome–independent pathway), leading to significant reduction in the VEGF levels (162). The endogenous physiologic role for 2-ME as an inhibitor of angiogenesis is not yet understood.

**Pigment epithelium-derived factor.** Because pigment epithelium-derived factor (PEDF), a noninhibitory member of the serpin superfamily, was identified to be responsible for the avascularity of ocular compartments in 1999, it is the most potent inhibitor of angiogenesis in the mammalian eye and is involved in the pathogenesis of angiogenic eye diseases, such as proliferative diabetic retinopathy (82, 163–167). It also has neurotrophic activity both in retina and in the central nervous system. PEDF expression is suppressed by hypoxia, and changes in PEDF correlate with the development of retinal neovascularization in animal models of hypoxic eye disease.

PEDF possesses several physiologic properties that make it a potentially important protein in the regulation of angiogenesis, in the neuronal cell survival, and in the protection of neurons from neurotoxic agents. Its antiangiogenic activity is selective, in that PEDF targets only new vessel growth but spares existing ones, and it is reversible. It is a protein that is highly up-regulated in the G₀ phase of early-passage cells compared with rapidly proliferating or senescent cells and thus is also linked to both cell cycle and cell senescence (168, 169).

Recent study highlights two beneficial effects of PEDF treatment on tumor growth and expansion. One is the suppression of tumor angiogenesis. Overexpression of PEDF was found to significantly inhibit melanoma growth and vessel formation in G361 nude mice xenografts (170). PEDF is also expressed strongly in normal murine kidney, and the loss of angioinhibitory activity may contribute to pathologic angiogenesis in Wilms’ tumor (171). In addition, PEDF may serve as a multifunctional antitumor agent in neuroblastomas, inhibiting angiogenesis while promoting the numbers of Schwann cells and differentiated tumor cells that in turn produce PEDF (172). The loss of PEDF expression was also detected in glioma progression (173). In PEDF-deficient mice, stromal vessels were increased and associated with epithelial cell hyperplasia (174). It is a key inhibitor of stromal vasculature and epithelial tissue growth in mouse prostate and pancreas. The other activity of PEDF is induction of Fasl-dependent apoptosis in tumor cells. PEDF up-regulates Fasl on endothelial cells. Expression of the essential partner of Fasl, Fas/CD95 receptor, was low on quiescent endothelial cells but greatly enhanced by inducers of angiogenesis, thereby specifically sensitizing the stimulated cells to apoptosis by inhibitor-generated Fasl. The antiangiogenic activity of PEDF, both in vitro and in vivo, was dependent on this dual induction of Fas and Fasl and the resulting apoptosis (82).

PEDF can be given therapeutically as a soluble protein or by viral-mediated gene transfer (175, 176). It is stable and nontoxic when injected systemically. Gene transfer of PEDF suppresses tumor vasculization and growth while prolonging survival in syngeneic murine models of thoracic malignancies. Gene transfer of PEDF using adeno-associated viral vectors also inhibited ischemia-induced neovascularization (177).

A recent study suggests that, in physiologic conditions, a critical balance between PEDF and VEGF exists, and PEDF may counteract the angiogenic potential of VEGF. Under oxidative stress, PEDF decreases, disrupting the angiogenic balance (178). This critical balance between PEDF and VEGF is important to prevent the development of choroidal neovascularization (163, 179). In addition, bone angiogenesis and matrix modeling may also be mediated both by dynamic intrapair between PEDF and by VEGF (180).

**PEX.** It has been shown that PEX, a noncatalytic COOH-terminal hemopexin-like domain of MMP-2, prevents binding of MMP-2 to integrin αvβ₃, thus inhibiting proteolytic activity on the cell surface and disrupting angiogenesis (181). Delivery of PEX by viral vectors resulted in suppressed endothelial cell invasion and formation of capillary-like structures, blocking of bFGF-induced MMP-2 activation and angiogenesis, and inhibition of tumor-induced angiogenesis and tumor growth in nude mice (182). PEX formation seems to depend on the stage of angiogenesis, reaching...
the maximum level during the late stage when the vessels are maturing (181).

**Platelet factor-4.** PF-4 is a protein released from platelet α-granules during platelet aggregation that has been shown to have antiangiogenic properties both in vitro and in vivo (183). Recombinant human PF-4 inhibits blood vessel proliferation in the chicken chorioallantoic membrane assay in a dose-dependent manner. PF-4 inhibits angiogenesis by associating directly with FGF-2, inhibiting its dimerization and blocking FGF-2 binding to endothelial cells. The inhibitory activities are associated with the COOH-terminal, heparin-binding region of the molecule (183) and more specifically in the short COOH-terminal segment (amino acids 47-70). A minor modification of this active segment of PF-4, an addition of an ERL, a critical domain present in proangiogenic chemokines, surprisingly elicits several times greater antiangiogenic potential than the original peptide. Thus, tailored PF-4 peptides represent a new class of antiangiogenic agents with a defined mode of action and a strong in vivo activity (184). Protein assays show that PF-4 and FGF-2 bind to adjacent or overlapping sites together covering a 12-kDa stretch of heparan sulfate, suggesting that these three components may form a ternary complex. This enables PF-4 to inhibit FGF-2-stimulated endothelial cell proliferation through heparan sulfate–dependent mechanism (185).

**Prolactin fragment.** The intact prolactin (23 kDa) is enzymatically cleaved in several different tissues to generate a 16-kDa (16K PRL) and a 8-kDa fragment (186). Although the intact prolactin has activities consistent with proangiogenesis, the generation of the 16K PRL fragment exposes a cryptic antiangiogenic activity (187, 188). The opposing angiogenesis activities of intact prolactin and NH2-terminal 16k PRL offers an insight into the body's mechanism to regulate angiogenesis (189). The mechanism by which such opposing action is regulated can in part be explained by differential regulation of MAPK signaling pathway (190). Additionally, the 16k PRL inhibits VEGF-induced activation of Ras in capillary endothelial cells (191).

**Tissue inhibitors of matrix metalloproteinases.** Tissue inhibitors of metalloproteinases (TIMP) suppress MMP activity and ECM turnover. In addition to their MMP inhibitory activity, TIMPs have pluripotent effects on cell growth, apoptosis, and differentiation (192–194). TIMP-2 inhibits angiogenic factor–induced endothelial cell proliferation in vitro and angiogenesis in vivo, independent of MMP inhibition. These effects require α5β1 integrin–mediated binding of TIMP-2 to endothelial cells. Further, TIMP-2 induces a decrease in total protein tyrosine phosphatase activity associated with β1 integrin subunit as well as dissociation of the phosphatase SHP-1 from β1 integrin. TIMP-2 treatment also results in an increase in protein tyrosine phosphatase activity associated with tyrosine kinase receptors FGFR receptor-1 and KDR (195).

**Tropomin 1.** Tropomin 1 (Tn 1) is a novel cartilage-derived angiogenesis inhibitor, which inhibits endothelial cell proliferation and angiogenesis in both in vivo and in vitro model systems. Tn 1 also inhibits metastasis of a wide variety of tumors in vivo. Tn 1 is a subunit of the tropomin complex, which along with tropomyosin is responsible for the calcium-dependent regulation of striated muscle contraction (196). Interestingly, Tn 1 is capable of inhibiting actomyosin ATPase, which might constitute for its angiogenesis, tumor growth, and metastasis inhibitory activity (196). Tn 1 inhibits both bFGF-stimulated and basal levels of endothelial cell proliferation probably via an interaction of Tn 1 with the cell surface bFGF receptor on capillary endothelial cells (197).

**Vasostatin.** Vasostatin, a NH2-terminal domain of human calreticulin inclusive of amino acids 1,180, is a potent angiogenesis inhibitor. It selectively inhibits endothelial cell proliferation and angiogenesis in response to stimulation from growth factors and suppresses tumor growth (198, 199). Burkitt, colon, and ovarian tumors in mice treated with vasostatin were smaller than tumors in control mice (150). Combination of vasostatin and IP-10 reduced tumor growth more effectively than each agent alone, but complete regression was not observed (200). Vasostatin specifically inhibits endothelial cell attachment to laminin and reduces subsequent endothelial cell growth induced by bFGF (201). Furthermore, gene therapy experiments with intramuscular delivery of vasostatin DNA is effective in the inhibition of angiogenesis and tumor growth in murine tumor models (202).

**Summary and Future Perspective**

Relative systemic levels of proangiogenic and antiangiogenic factors likely govern tumor progression by regulating the “angiogenic balance.” Conversion of dormant carcinomas to an invasive malignant carcinomas is considered to involve a shift in favor of enhanced angiogenesis potential. Influenced by oncogenes and tumor suppressor genes, disruption of the “angiogenic checkpoint” via increase in angiogenic factors, such as VEGF, or decrease in the physiologic levels of endogenous inhibitors of angiogenesis, like TSP-1, tumstatin, and endostatin, could represent an important step in the progression of cancer. Currently, at least 27 different protein and small molecules are known to exist in the body that function as inhibitors of angiogenesis. Therefore, it is quite possible that genetic control of the physiologic levels of endogenous inhibitors of angiogenesis might constitute a critical line of defense against the conversion of dormant neoplastic events into a malignant phenotype of cancer.

How these endogenous inhibitors orchestrate the formation of a barrier to counteract the effects of physiologic levels of proangiogenic growth factors and cytokines is not yet understood. For endogenous inhibitors that need to be generated via protein synthesis of larger precursor proteins, regulation of enzymatic activity also likely plays a key role in the formation of the angiogenic barrier. Further studies are needed to address the function of physiologic levels of these 27 inhibitors in controlling cancer progression. It is conceivable that genetic defects reflected by loss-of-function single nucleotide polymorphisms in the sequence of the protein inhibitors or enzymes that generate angiogenesis inhibitors could determine how fast a set of neoplastic events switch from a nonlethal lesion to a malignant and angiogenic tumor.

Lastly, the potential therapeutic application for these endogenous inhibitors of angiogenesis is being considered in the clinic and early results from clinical trials with angioskatin, endostatin, TSP-1 (ABT-510), and 2-ME (Panzam) suggest that more laboratory studies are required to better understand the mechanism of action associated with each of these drug candidates (203–209). Such continuing efforts will enable us to design more relevant and targeted clinical trial guided by the inherent antiangiogenic mechanism of each particular inhibitor.

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Endogenous Inhibitors of Angiogenesis

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