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Cellular Actions and Signaling by Endostatin

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Abstract

The malignant transformation of a normal cell into a cancer cell requires no vasculature. Growth of solid tumors, however, requires angiogenesis to provide oxygen and nutrients to support cell proliferation. The switch from an avascular to a vascular phenotype is typically associated with acceleration of tumor growth. Antiangiogenic therapy, starving a tumor of its blood supply, is an attractive addition to the anticancer armamentarium. Animal tests of antiangiogenic therapy have shown remarkable potential. Initial human trials have proven antiangiogenic therapy to be remarkably nontoxic. Numerous antiangiogenic agents have been isolated as proteolytic fragments of endogenous polypeptides of the extracellular matrix. Endostatin was the first such antiangiogenic protein described and its potent antitumor effects in mice have generated wide interest. This review summarizes recent advances in endostatin biology and highlights new results on the cellular and subcellular mechanisms of endostatin action.

Keywords

angiogenesis; cancer; extracellular matrix; growth factors; receptor

I. INTRODUCTION AND BACKGROUND

A. Historical Perspective

The first angiogenesis inhibitor was discovered almost 25 years ago (Brem and Folkman, 1975). In the subsequent years the number of molecules discovered to exhibit antiangiogenic function has risen exponentially. The ongoing discovery of antiangiogenic molecules has occurred in three phases. The first phase of discovery yielded small antiangiogenic molecules such as protamine (Taylor et al., 1982), TNP-470 (Ingber et al., 1990), and carboxyaminotriazole. The second phase was characterized by discovery that circulating polypeptide growth factors/cytokines such as platelet factor 4 (Maione et al., 1990),

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interferon-α (Sidky et al., 1987), and thrombospondin (Tolsma et al., 1993) could also exhibit antiangiogenic activity. Finally, in the mid- to late 1990s fragments of proteins (themselves inactive as antiangiogenic molecules) were discovered to be antiangiogenic. Endostatin, the 20-kDa C-terminal NC1 domain of collagen XVIII, belongs in this last category (O'Reilly et al., 1997). Endostatin is only one of a rapidly expanding group of proteolytic fragments of coagulation pathway and matrix proteins that exhibit antiangiogenic activity (Kuroi et al., 2001b). Table 1 presents a current list of antiangiogenic proteins, which are fragments of larger proteins.

Endostatin was discovered by screening chromatographic fractions isolated from supernatants of a hemangioendothelioma (EOMA) cell line for the ability to inhibit angiogenesis and tumor growth (O'Reilly et al., 1997). Repeated cycles of endostatin therapy in mice were followed by tumor dormancy, which, surprisingly, persisted after cessation of endostatin treatment (Boehm et al., 1997). Media coverage increased public and scientific interest in rapid application of endostatin and other antiangiogenic substances to treatment of human cancers. (Harris, 1999; Rowe, 1999). Phase I clinical trials have, at the time of this writing, concluded with confirmation of no evident toxicity and with modestly encouraging degrees of tumor stasis or regression; additional trials have been planned to investigate responses to different doses and schedules (Herbst et al., 2001; Mundhenke et al., 2001).

B. Angiogenesis

Angiogenesis, the formation of new capillaries by sprouting from existing ones, is a fundamental process for generating new blood vessels. It occurs primarily during embryonic development (Folkman, 1995b; Hanahan et al., 1996). In the adult, angiogenesis occurs during wound repair, hair growth, the estrus cycle, and pregnancy. Physiological angiogenesis is tightly regulated. When the balance between local inhibitors and inducers, however, is lost, then dysregulated angiogenesis contributes to pathological conditions, such as rheumatoid arthritis, psoriasis, diabetic retinopathy, and hemangiomas (Folkman, 1995a). The link between tumor growth, metastasis, and angiogenesis was established by Judah Folkman when he discovered that neovascularization accompanied growth of tumors beyond a few millimeters in diameter (Folkman, 1971). To test the concept that blocking angiogenesis might be useful for treating solid tumors required the development of assays for angiogenesis to identify pro- and antiangiogenic molecules. In vitro angiogenesis assays can be classified into three broad categories: namely, cell culture, organ culture, and embryo culture. The advantages and disadvantages of these assays are discussed elsewhere (Jain et al., 1997; Auerbach et al., 2000). In vivo assays for angiogenesis include mouse corneal, intradermal angiogenesis, matrigel plug, and chick chorioallantoic membrane (CAM) assays. The currently accepted standard for evaluation of the antitumor effect of antiangiogenic proteins, however, is the syngeneic orthotopic mouse tumor model.

II. ENDOSTATIN ACTIONS

The discovery of endostatin by O'Reilly and colleagues in the Folkman laboratory arose out of Folkman's clinical observation that surgical removal of a primary tumor often accelerates growth of distant metastatic foci. He hypothesized that the primary tumor might secrete circulating angiogenesis inhibitors of extended half-life, which serve to suppress growth of distant metastases (Folkman, 1971). Folkman and colleagues then identified several primary mouse tumor types, including EOMA, which appeared to suppress growth of their metastases. They next devised a strategy for biochemical purification of the putative endogenous inhibitors secreted by the primary tumor from mouse serum or urine (Chen et al., 1995). This strategy led to identification of angiostatin, an antiangiogenic proteolytic fragment of plasminogen (O'Reilly, 1997). Subsequent purification protocols used

conditioned tumor cell supernatant as starting material. Endostatin was isolated from the EOMA cell supernatant (O'Reilly et al., 1997).

A. In Vitro Effects

Endostatin is a 184-amino acid C-terminal monomeric fragment of the noncollagenous domain of type XVIII collagen. Recombinant endostatin produced in baculovirus infected insect cells and in bacteria inhibited endothelial cell proliferation, but in initial studies had no effect on tumor cell proliferation in vitro. The initial conclusion that endostatin's antitumor activity was secondary to antiangiogenic activity was primarily based on in vitro antiproliferative effects in endothelial cells (O'Reilly et al., 1997). Subsequently, recombinant endostatin produced in yeast was also found to promote apoptosis of endothelial cells but not nonendothelial cells (Dhanabal et al., 1999b). Another mechanism for endostatin's antiproliferative effect is blockade of endothelial S-phase entry, leading to cell cycle arrest (Dhanabal et al., 1999c; Hanai Ji et al., 2002; Shichiri et al., 2001). However, the proapoptotic and growth inhibitory effects of endostatin in tissue culture are observed under low serum conditions.

Endostatin also causes dose-dependent inhibition of VEGF-induced migration of human umbilical vein endothelial (HUVE) cells (Karumanchi et al., 2001; Yamaguchi et al., 1999). Both human and mouse endostatin protein inhibit migration of human endothelial cells (Shichiri, Hirata, 2001; Yamaguchi et al., 1999). Interestingly, the trimeric NC1 domain of type XVIII collagen is promigratory, and this motility-inducing activity of type XVIII NC1 domain was blocked by the physiologically cleaved endostatin monomer (Kuo et al., 2001b). These authors speculate that the proteolysis step which, generate endostatin represents a negative feedback mechanism.

One caveat in the interpretation of endostatin's antimigration effects is that the initial report (Dhanabal et al., 1999a) of endostatin's inhibition of endothelial cell migration was performed with ECV304 cells. These have since been shown to be T24 bladder carcinoma cell derivatives that express endothelial markers (Dirks et al., 1999; Suda et al., 2001). However, several groups in addition to our own confirmed the antimigratory effects of endostatin in HUVE cells (Taddei et al., 1999; Yamaguchi et al., 1999; Karumanchi et al., 2001). Endostatin was recently reported to induce plasminogen activation and concurrent loss of focal adhesion and actin stress fibers in human endothelial cells (Wickstrom et al., 2001). Endostatin was also shown to affect FGF-2–induced actin, FAK, paxillin, and β-catenin, suggesting that endostatin can disturb cell–matrix interaction (Dixelius et al., 2002). These cytoskeletal alterations in endothelial cells may mediate in part the antimigratory function of endostatin.

B. In Vivo Effects

1. Antitumor Effects of Endostatin—Recombinant endostatin preparations inhibited metastases, angiogenesis, and the growth of primary tumors when administered to mice previously injected with syngeneic tumor cells. Among the susceptible tumors were Lewis lung carcinoma, T241 fibrosarcoma, B16F10 melanoma, and EOMA (O'Reilly et al., 1997). These and other tumor types susceptible to endostatin are summarized in Table 2. Decreased tumor growth in endostatin treated mice was explained by increased apoptosis without change in proliferation rate (O'Reilly et al., 1997). The mechanism of inhibition of tumor growth in vivo, however, remains unknown. Recently, endostatin was shown to inhibit endothelial and tumor cell invasion by blocking the activation and catalytic activity of matrix metalloproteinase (MMP-2) (Kim et al., 2000), an additional mechanism for explaining the potent antiangiogenic and antitumor activities of endostatin.

Murine tumors regressed to dormant microscopic lesions when treated systemically with murine endostatin (O'Reilly et al., 1997). When endostatin therapy was stopped, the tumors regrew, but upon repeated therapy the tumors again regressed. More remarkably, multiple treatment cycles rendered the tumors dormant. They failed to regrow after stopping therapy (Boehm et al., 1997). This study provided the first evidence that tumors in mice do not develop resistance to antiangiogenic therapy (Kerbel, 1997). Recently, a phenomenon known as "vasculogenic mimicry" has been described in which a uveal melanoma cell line can mimic endothelial cells in forming tubular structures in vitro (Maniotis et al., 1999). If vasculogenic mimicry by tumor cells occurs in vivo, then a form of resistance to antiangiogenic therapy might result (Folberg et al., 2000; McDonald et al., 2000). Indeed, ovarian cancer (Sood et al., 2001) and breast cancer (Hendrix et al., 2000) cells have shown similar tendencies. Additional indirect evidence has been provided by microarray transcriptome comparisons of highly aggressive and nonaggressive tumor cells, in which aggressive cells exhibit up-regulation of endothelial marker genes (Hendrix et al., 2001). However, even if these tumor cells can mimic endothelial cells, endothelial cell growth and remodeling must occur at the junction between postulated tumor cell-lined vessels and the endothelial cells of the host vasculature. This junction might represent a point of susceptibility to antiangiogenic therapy. Moreover, not all cells lining the blood vessels within a tumor are likely to be of tumor origin. It is worth noting that the susceptibility to endostatin of ECV304 bladder tumor cell migration (Dhanabal et al., 1999a) may itself exemplify such vasculogenic mimicry being overcome by an antiangiogenic agent.

2. Other In Vivo Effects of Endostatin—Mouse endostatin produced in *Pichia pastoris* produced dose-dependent inhibition of angiogenesis induced by FGF-2 or by VEGF in a CAM assay (Dhanabal et al., 1999a). Sasaki et al. also reported robust inhibition of FGF-2-induced CAM angiogenesis by endostatin produced in mammalian cells, but a mild inhibitory effect on VEGF-induced CAM angiogenesis (2000). The contrasting results with VEGF may be attributable to differential post-translational modifications between murine endostatins produced in *P. pastoris* and in mammalian cell culture. Interspecies differences in endostatin amino acid sequence may also lead to differences in biological activity. Murine but not human endostatin shows antiangiogenic activity in an ex vivo rat aortic ring assay, suggesting that this model may have species specificity. The human form, however, shows biological activity against human vascular tissue in a human saphenous vein assay (Kruger et al., 2000a).

3. Effect of Endostatin on Physiological Angiogenesis—A concern with antiangiogenic therapy is that it may have an effect on "physiological angiogenesis." Two studies have investigated the effect of endostatin on wound healing; one reported no effect on wound healing in endostatin-treated mice (Berger et al., 2000), whereas another reported subtle changes (Bloch et al., 2000). Different methods of evaluation of wound healing likely account for this discrepancy. A recent report has investigated the role of endostatin in gastric ulcer healing and hypothesized a role for the platelet released endostatin in modulating gastric ulcer healing (Ma et al., 2001). A recently concluded clinical trial, however, showed that endostatin treatment did not perturb wound healing (Mundhenke et al., 2001). These findings raise important questions about the mechanistic differences between "physiological" angiogenesis and tumor angiogenesis.

C. Endostatin Production

1. Recombinant Endostatin Production Systems—An initial concern with the first report of endostatin's antitumor activity in vivo was that an insoluble preparation of bacterially produced endostatin was used. Subsequently, a soluble preparation of endostatin generated in *P. pastoris* was reported to show similar antitumor effect in a xenograft model

of renal cell carcinoma (Dhanabal et al., 1999a; Dhanabal et al., 1999c). Several other groups have since produced endostatin in *Escherichia coli* (Huang et al., 2001) and in mammalian cells (Sasaki et al., 1998), taking care to avoid endotoxin contamination. The recombinant proteins from either source have exhibited similar efficacy in both syngeneic and xenograft tumor models (Yoon et al., 1999; You et al., 1999; Perletti et al., 2000; Yokoyama et al., 2000b; Boehle et al., 2001).

2. Endogenous Generation of Endostatin—The first evidence that endostatin could be released from the NC1 domain of collagen XVIII by proteolytic cleavage was presented by Sasaki et al. (1998). Importantly, the proteolytic release of endostatin can occur through several pathways, leading to a switch from a matrix-associated to a more soluble, circulating form. Recently, cathepsin L (Felbor et al., 2000) and elastase (Wen et al., 1999) have been implicated in the generation of endostatin from collagen XVIII. On the basis of the results that the trimeric NC1 domain was partially cleaved by endogenous proteolysis into several monomeric polypeptide fragments related to endostatin, these authors concluded that the NC1 domain consists of an N-terminal association region, a central protease-sensitive hinge region, and a stable C-terminal endostatin domain. Finally, proteinases such as cathepsins L and B have also been implicated in endostatin degradation, suggesting another potential regulatory step in angiogenesis (Ferreras et al., 2000).

D. Endostatin Fragments and Antibodies In Vivo

Circulating fragments of human endostatin were first detected and isolated from human plasma (Standker et al., 1997). These fragments, however, were truncated versions of endostatin devoid of antiproliferative activity. The same group later isolated glycosylated forms of human plasma endostatin (John et al., 1999). In addition to endostatin, they also found restin, a C-terminal NC1 domain of type XV collagen that shares 60% amino acid identity with endostatin, whose biological activity as antiangiogenic molecule had been previously described (Ramchandran et al., 1999).

Circulating serum endostatin levels have been used as a diagnostic marker for clinical studies. Curiously, elevated levels of serum endostatin (probably reflecting collagen XVIII turnover) have been measured in patients with hepatocellular carcinoma (Musso et al., 2001; Yamagata et al., 2000), vulvar cancer (Hefler et al., 1999), head and neck squamous cell carcinoma (Homer et al., 2000), clear cell renal cancer (Feldman et al., 2000b), breast cancer (Kuroi et al., 2001a), soft-tissue sarcoma (Feldman et al., 2001b), endometrial hyperplasia, endometrial cancer (Shaarawy et al., 2001), and endostatin were also detected in the serum and brain tumor tissue from a patient with multifocal glioblastoma suggestive of a humoral immune response to endogenous endostatin production (Ratel et al., 2000). The first study of endostatin as a marker for predisposition to cancer reported the association of an endostatin polymorphism with predisposition to development of prostatic adenocarcinoma (Iughetti et al., 2001). The functional effect of this endostatin polymorphism, however, remains to be defined. Besides cancer, endostatin levels are also elevated in a nonmalignant but “proliferative” disease such as rheumatoid arthritis (Hebbar et al., 2000) and diabetic retinopathy (Funatsu et al., 2001).

III. MECHANISM OF ACTION

Endostatin’s antiangiogenic function can be envisioned via several mechanisms. First, endostatin could interfere with proangiogenic signal initiated by VEGF or FGF-2. This could be achieved by competing with either VEGF or FGF-2 for binding to their respective receptors or by directly interacting with these molecules, thus rendering the growth factors incapable of receptor binding or phosphorylation. Second, endostatin could act via its own receptor(s) transmitting either an agonistic antiangiogenic signal or intercepting a

proangiogenic signal initiated by proangiogenic growth factors. Third, endostatin might alter the appropriate assembly of type XVIII collagen, thereby affecting vascular basement membrane composition and endothelial survival (Bjorn R. Olsen, Harvard Medical School). The latter does not account for the effects of endostatin on endothelial cell proliferation or migration (unless one invokes production of matrix by endothelial cells during the assay period) but may be important in vivo.

Initial experiments investigated the first mechanism above. Competitive binding experiments with iodinated ^{125}I-VEGF or ^{125}I-FGF-2 in the presence of increasing concentrations of endostatin indicated that endostatin did not change VEGF or FGF-2 binding to their cell surface receptors (unpublished data, R. Ramchandran and V.P. Sukhatme). Endostatin's inability to compete for FGF-2 binding has also been demonstrated in situ (Chang et al., 1999). Similarly, endostatin did not inhibit the ability of FGF-2 and VEGF to promote phosphorylation of their receptors (Knebelmann et al., 1999).

A. Cell Surface Receptors

To explain endostatin's antiangiogenic function several groups have pursued the second mechanism and searched for endostatin-binding proteins on the cell surface. Three articles have been published on this subject to date. The first article reported that human endostatin interacts with α_5 and α_v integrins on human endothelial cells (Rehn et al., 2001). This article did not assess the affinity of this interaction. However, the authors showed that the endostatin-integrin interaction was functional in vitro in migration and cell survival assays. Immobilized and soluble endostatin showed differential effects on HUVEC migration assays. Antibodies to α_5 and not $\alpha_v\beta_3$ blocked migration of HUVE cells toward immobilized endostatin (1–2 μM). Soluble endostatin (50 nM), however, inhibited cell migration on immobilized gelatin, a $\alpha_v\beta_3$ integrin-dependent activity. The authors conclude that immobilized endostatin promotes and soluble endostatin inhibits migration in an integrin-dependent and integrin subtype-specific manner.

In the second report, we used an alkaline phosphatase tagged endostatin (AP-ES) for generating cell-specific binding curves and as a tool for expression cloning. Our expression cloning strategy identified glypicans, a major class of cell surface heparan sulfate proteoglycans, as the low-affinity cell-surface binding partners for endostatin (Karumanchi et al., 2001). Interestingly, AP-ES binding to endothelial cells revealed two affinities by Scatchard analysis. The lower affinity K_d ranged from 200 to 500 pM and the higher affinity K_d ranged from 18 to 36 pM. Glypican binding was shown to represent the lower affinity component. Additional biochemical and genetic analysis demonstrated that the heparan sulfate glycosaminoglycan moieties of glypicans were critical for endostatin binding and that there exhibited a glycan sequence specificity to this interaction. Moreover, functional relevance for the endostatin-glypican interaction was demonstrated by antisense experiments. HUVE cell migration experiments showed that endostatin inhibited endothelial cell migration in response to $VEGF_{165}$ and $VEGF_{121}$ the latter not requiring heparan sulfate proteoglycans for its action. However, in cells infected with glypican-1 antisense complementary DNA (cDNA), endostatin was unable to block $VEGF_{121}$-induced migration. This antisense experiment suggested that glypicans were necessary for the antimigratory action of endostatin on endothelial cells.

The third report used phage-display technology to identify conformationally constrained peptides that might mimic the binding domains of proteins with which endostatin interacts (MacDonald et al., 2001). The endostatin binding phage expressed a novel peptide sequence (E37) without homology in the database. Crude antiserum raised against E37 coupled to KLH was used to screen a FGF-2–stimulated HUVEC cDNA expression library in phage λ. The screen yielded only cDNAs encoding human tropomyosin 3, an intracellular actin-

binding protein, and the screening antibody indeed recognized two isoforms of tropomyosin on immunoblot. The binding affinity of E37 for endostatin, however, as measured by surface plasmon resonance, was reported to be only 100 μM, and did not achieve saturation. Furthermore, although 2 μM Alexa-labeled endostatin appeared partially to colocalize with tropomyosin by immunofluorescence microscopy of permeabilized cells, 100 μM E37 failed to compete the strong Alexaendostatin labeling of the plasma membrane, the nucleus, and the endoplasmic reticulum. Nonetheless, a 350-fold molar excess of E37 blocked 84% of the tumor-growth inhibitory activity of human endostatin in a B16-BL6 meta-static melanoma model. Thus E37 recognizes a mimotope for an endostatin-binding site, but the identity of that binding site as tropomyosin remains to be ascertained.

At this time, only glypicans and integrins have been proposed as cell surface receptors for endostatin. Only glypicans exhibit apparent binding affinities consistent with the potencies of endostatin-mediated inhibition of endothelial cell migration, proliferation, and enhancement of apoptosis. Interestingly, removing glypicans from the cell surface by antisense ablates the endostatin binding to the higher affinity component suggesting a cooperative interaction between the two moieties. We currently favor a two-receptor model for endostatin's binding to endothelial cells. In this model, glypicans acts as the endostatin coreceptor along with the high-affinity signaling receptor. We have not ruled the possibility out, however, that both the high- and low-affinity site resides on glypican and the GPI anchor of glypican can transduce a signal into the cell. Interestingly, GPI anchors are known to transduce signals into the cells (Davy et al., 1999). The possibility that the GPI anchor in glypican possess similar function in transducing endostatin's antiangiogenic signal is currently being investigated in this laboratory.

B. Intracellular Signaling

Only in the last year has progress been reported in defining endostatin signaling at a cellular level. Several pathways, however, have been implicated in endostatin action, and no unified picture of endostatin signaling has yet emerged. Endostatin increases apoptosis in endothelial cells by down regulation of antiapoptotic Bcl-2 and Bcl-XL protein expression and up regulation of caspase-3 activity (Dhanabal et al., 1999b). Endostatin-induced tyrosine kinase signaling has been implicated to regulate endothelial cell apoptosis (Dixelius et al., 2000). In this study, murine brain endothelial cells overexpressing the adaptor protein Shb display elevated apoptosis levels in response to endostatin in the presence of FGF-2. Endostatin treatment for 10 minutes or 24 hours induced tyrosine phosphorylation of Shb and the formation of a multiprotein complex. A Shb SH2 fusion protein precipitated a 125-kDa phosphotyrosyl protein in endostatin-treated cells. Intracellular Ca^{2+} signaling has been reported for both endostatin and angiostatin (Jiang et al., 2001). In this report, endostatin-induced peak elevations in intracellular Ca^{2+} in endothelial cells exceeded those in epithelial and tumor cells. The elevation in Ca^{2+} derived partially from inositol-triphosphate-sensitive intracellular Ca^{2+} stores and partially from entry of extracellular Ca^{2+} across the plasma membrane. The Ca^{2+} signal was partially sensitive to pertussis toxin, and was diminished in cells pretreated overnight with either VEGF or FGF-2.

A recent report has noted down-regulation of many genes in exponentially growing endothelial cell treated with endostatin in reduced serum conditions (Shichiri, Hirata, 2001). Among these genes were many apoptosis and growth-related genes. The authors argued that suppression of cell proliferation and pro-apoptotic genes contribute little to the antiangiogenesis process because endostatin induces neither apoptosis nor growth inhibition under normal serum conditions. The antimigratory effect of endostatin, however, was present even under serum-supplemented conditions, and over-expression of the c-*myc* gene into the endothelial cells abrogated the antimigratory effect of endostatin. The authors concluded that rapid down-regulation of genes by endostatin causes endothelial cell cycle

arrest or induce apoptosis but potently inhibits migration of endothelial cell, at least in part via inhibition of c-*myc* expression. We have recently obtained evidence that cyclin D1 is a target gene for endostatin and that an LEF1 site in the cyclin D1 promoter is critical for mediating endostatin's repressive effect (Hanai Ji et al., 2002). Taken together these results suggest that multiple signaling pathways were triggered in endothelial cells by endostatin.

C. Structure-Function Studies of Endostatin

A recombinant mouse endostatin expressed in 293-EBNA cells was crystallized at 1.5 Å resolution (Hohenester et al., 1998). The structure revealed a compact fold distantly related to the C-type lectin sugar recognition domain. An extensive surface cluster of 11 basic amino acid (arginine) residues was predicted to represent the moderate affinity (Kd 300 nM) binding site for heparin. A second group reported a 3 Å crystal structure of human endostatin overexpressed in a murine myeloma cell line (Ding et al., 1998). Atomic absorption spectroscopy indicated that zinc was a constituent of both human and mouse endostatin in solution. Zinc-dependent dimers were also observed in the human endostatin crystals. The presence of the zinc site at the polypeptide's amino terminus, proximal to the precursor cleavage site, suggested a role for zinc in the activation of the antiangiogenic activity of endostatin after cleavage (Ding et al., 1998). Mouse endostatin was recrystallized at pH 8.5 because the original crystals were generated at pH 5.0 (Hohenester et al., 2000). Two structures were present in the crystals. One showed metal ion coordination similar to that observed in the crystal structure of human endostatin. The second structure possessed a different metal ion coordination center. Moreover, endostatin mutants lacking the zinc binding domains are still able to block migration of endothelial cells and inhibit tumor growth in vivo (Sim et al., 1999; Yamaguchi et al., 1999). Thus, zinc likely plays no critical role in endostatin activity but has been postulated to stabilize the structure of endostatin.

The first engineered mutations of endostatin were planned to explore the role of surface histidine residues, based on the human endostatin crystal structure. When compared to wild-type endostatin, the three mutants H132A/134A, H142A, and D208A were unable to cause regression of Lewis lung carcinomas in antitumor studies (Boehm et al., 1998). A second set of mutation studies analyzed the role of the C-terminus (Dhanabal et al., 1999a). The EM2 mutant with a 17-amino acid C-terminal deletion was inactive in vivo. The EM1 mutant with a 9 deletion retained antitumor activity in vivo. These mutants, however, were expressed in *E. coli*, and the precipitated protein was injected in the mice. (The residues are numbered with reference to the first amino acid H132 of the NC1 collagen type XVIII domain.)

A series of soluble endostatin point mutants was generated in mammalian cells and tested in VEGF-induced endothelial cell migration assay (Yamaguchi et al., 1999). All the mutants reported in this study, retained function. Another report produced recombinant mouse endostatin in mammalian cells and carried out extensive alanine scanning mutagenesis. This study demonstrated that 4 arginines clustered in the three-dimensional structure (R155, 158, 184, and 270) and at a second site (R193, 194) are essential for endostatin binding to heparan sulfates and sulfatides in solid-phase assays (Sasaki et al., 1999). However, the importance of endostatin's heparin binding activity to its antiangiogenic binding activity also remains controversial.

Prompted by assignment of surface residues in the crystal structure of endostatin, the double mutant F162A/F165/A (ES3.1) (Karumanchi et al., 2001) was expressed in mammalian cells and in *P. pastoris*. Sasaki et al. (1998) had previously shown that the single mutants F162A and F165A retained heparin binding capability. However, the double mutant was unable to block VEGF-induced migration of endothelial cells (Karumanchi et al., 2001). Interestingly, the double mutant and wild-type endostatin polypeptides were indistinguishable in their shared elution from a heparin column at 0.2–0.3 M NaCl. This argues that heparin binding

does not equate with heparan sulfate binding because ES3.1 bound to heparin, yet was inactive as a cell migration inhibitor. Whether ES3.1 can inhibit angiogenesis induced by FGF-2 or VEGF in a CAM is yet to be investigated.

IV. NONENDOTHELIAL ACTIONS OF ENDOSTATIN

The early hypothesis of endothelial specificity of endostatin's action has recently required revision. Deletion of the collagen XVIII homologue *cle-1* NC1 domain of *Caenorhabditis elegans* results in cell migration and axonal guidance defects (Ackley et al., 2001). This phenotype was rescued by ectopic expression of the NC1/endostatin trimeric domain, suggesting a role for endostatin in cell migration during neurogenesis in *C. elegans*. We have also recently studied the effects of endostatin RNA on *Xenopus* embryogenesis and have observed developmental abnormalities consistent with impaired Wnt signaling. Furthermore, we have demonstrated that endostatin inhibits LEF transcription by causing degradation of β-catenin and preventing β-catenin translocation to the nucleus (J. Hanai, V. P. Sukhatme, S. Sokol, manuscript in review).

A recent study has indicated that endostatin inhibits renal epithelial cell branching process formation and migration induced by either hepatocyte growth factor or epidermal growth factor (Karihaloo et al., 2001). These data suggest that endostatin regulates branching morphogenesis of renal epithelial cells, a process that precedes tubulogenesis. Endostatin also inhibits branching of the explanted murine ureteric bud, and a neutralizing antibody to endostatin rescues this effect. This study concluded that endostatin is made at the tips of ureteric buds and that endostatin may play a role in the regulation of ureteric bud arborization (Karihaloo et al., 2001).

All these results suggest that collagen XVIII/endostatin may play key roles in the vertebrate cell development pathways. Col XVIII(−/−) mice, however, appeared grossly normal (Fukai et al., 2002). A lack of renal or brain phenotype may suggest compensation by molecules similar to endostatin such as restin (Ramchandran et al., 1999). However, closer observation of angiogenesis in the developing eye in the knockout mice revealed incomplete regression of the hyaloid vessel and a "sticky iris" effect. This ocular phenotype resembles the vitreoretinal degradation and retinal detachment problems of Knobloch syndrome that results from mutations that influence splicing in human collagen XVIII (Sertie et al., 2000).

V. ANTIANGIOGENIC THERAPY

Therapy for cancer typically has targeted tumor cells, whereas antiangiogenic therapy focuses on the endothelial cells of tumor blood vessels. Several benefits of antiangiogenic therapy for cancer over conventional therapy can be envisioned. Drug resistance is less likely to develop because this therapy targets the genetically stable endothelial cell population. Less toxicity is expected with this treatment as compared to radiation or chemotherapy because angiogenesis is not a widespread phenomenon in the adult. Indeed, most animals appear normal after antiangiogenic therapy with no side effects. Antiangiogenic agents may work on many different primary solid tumors and in metastatic disease because tumor growth and metastases are angiogenesis-dependent. Synergy with other modalities of treatment such as radiation and chemotherapy has been shown (Mauceri et al., 1998; Hanna et al., 2000), which may help in reducing dosage and in minimizing side effects of radiotherapy and cytotoxic therapy. Specific markers on endothelial cells, such as integrins, may help in targeting the tumor vasculature with antiangiogenics. Because one endothelial cell is estimated to feed 50–100 tumor cells, amplification of the antitumor effect is expected. Finally, because several antiangiogenic proteins are fragments of endogenous molecules, immunogenicity appears only of modest risk.

There are, however, some projected limitations of antiangiogenic therapy. Such therapies are likely to take longer to act than radiation or chemotherapy and may only be cytostatic in the short-run. Large amounts of antiangiogenic proteins are needed to regress or hold tumor growth. All in vivo experiments performed to date have been on mouse vasculature. Whether these proteins work on human vasculature is yet to be determined. For protein therapies in general, oral administration is not an option. Most importantly, little is known about the mechanisms by which these proteins act. The direct correlation between the antimigration or antiproliferative or proapoptotic effects of the antiangiogenic molecules contributing to the in vivo antitumor effect remains poorly understood.

Some of these limitations can be addressed by gene transfer strategies. Both viral and nonviral vectors have been used to transfer the endostatin gene in vivo. Adenovirus (Feldman et al., 2000a; Kuo et al., 2001a; Sauter et al., 2000), adenoassociated virus (Nguyen et al., 1998), retroviruses (Feldman et al., 2001a; Scappaticci et al., 2001), Semliki forest viruses (Yamanaka et al., 2001), and lentivirus (Shichinohe et al., 2001) have been used to deliver the endostatin gene, in each case leading to inhibition of tumor growth in mice. Several nonviral methods of endostatin gene transfer have also been successful. Intramuscular administration to mice of the endostatin gene complexed with polyvinylpyrrolidone (“naked DNA”) resulted in inhibition of both primary and metastatic tumors (Blezinger et al., 1999). Liposomes complexed with endostatin cDNA have also been used to inhibit lung metastases in mice (Chen et al., 1999). Naked plasmid DNA expressing the endostatin gene administered intratumorally has also been reported to inhibit tumor growth (Ding et al., 2001; Szary et al., 2001).

Recently, two groups have described the use of alginate encapsulated endostatin-producing cells in the treatment of brain tumors (Joki et al., 2001; Read et al., 2001b). The endostatin-transfected cells encapsulated in alginate maintained endostatin secretion for at least four months after intracerebral implantation in rats. Rats that received transplants of BT4C glioma cells, together with endostatin-producing capsules (0.2 μg/mL per capsule), survived 84% longer than the controls. Intravital microscopy showed the antivascular and antitumor effects of endostatin delivered by the alginate capsules (Read et al., 2001a). All these results suggest that endostatin gene transfer may become a viable alternative to protein therapy. Recent evidence, however, has disputed the efficacy of endostatin gene therapy studies (Marshall, 2002). Two groups have independently reported that endostatin expressed in either hematopoietic stem cells (Pawliuk et al., 2002) or by gene transfer (Eisterer et al., 2002) does not inhibit blood vessel or tumor growth. The authors report that levels as high as 750 ng/mL of endostatin were obtained in the systemic circulation and yet no effect was seen. A similar study by the NCI counteracts these results and shows that when endostatin secreting tumor cells were implanted into mice, only those implants expressing the highest amounts of endostatin (1 μg/mL or more) were inhibited in their growth (Feldman et al., 2001a). One reason for this discrepancy could be that the protein tested by Eisterer et al. and Pawliuk et al. (in vitro assays) had a different conformation than the one generated in vivo in the mouse. Others speculate, however, that high amounts of endostatin could aggregate because of the innate collagen XVIII characteristic and hence render the protein inactive. Whatever the reason, it is clear that the efficacies generated by protein therapy and gene therapy for endostatin is paradoxical to say the least. Additional experiments will clarify this issue.

VI. CLINICAL TRIALS

Since the first clinical trial of an antiangiogenic drug TNP-470 began in 1992, the list of antiangiogenic drugs entering clinical trial has steadily increased (Kruger et al., 2000b). For

a current list of angiogenesis inhibitors being evaluated in phase I, II, and III clinical trials, refer to the Angiogenesis Foundation Web site (http://www.angio.org).

The clinical development of antiangiogenic compounds faces novel challenges. At least three settings for administration of such therapy can be envisioned. First, for advanced disease, the drugs would need to be given chronically with the goal of stabilizing tumor growth and perhaps regress it. Second, if antiangiogenics were used as radiation or chemo sensitizers, short courses would be adequate. Third, antiangiogenics could be administered after chemotherapy, radiation, or surgery in the setting of minimal residual disease to lengthen time to recurrence. Conventional concepts of dose-limiting toxicity and maximum tolerated dose must be reevaluated, because most antiangiogenic proteins are given at high doses in mice without apparent toxicity. Parameters used to describe efficacy of therapy also need to be changed. Acceptable endpoints established for conventional chemotherapy will need revision, because prolonged tumor dormancy replaces tumor killing. Additional challenges associated with clinical trials for antiangiogenic therapy have been reviewed by Kerbel (Kerbel, 2001).

Most antiangiogenic therapy is cytostatic when used as a single therapy agent. The absence of tumor shrinkage or regression in a phase I clinical trial may be regarded as a failure by oncologist used to dealing with cytotoxic agents. Yet, stable disease may be a more important outcome for evaluating the efficacy of a phase II clinical trial than tumor shrinkage. The disappointment from the phase I endostatin clinical trial was the lack of major regression with endostatin therapy. The objective of a phase I trial, however, which demonstrates a lack of toxicity, was met by endostatin and by angiostatin. Despite the lack of regression of cancer in the majority of patients, 3 of the 50 patients showed some regression after endostatin therapy. More recently, the mode of endostatin administration has been shown to be important for efficacy of treatment. Continuous administration of endostatin by intraperitoneally implanted osmotic pumps improved the efficacy of endostatin in a mouse xenograft tumor model (Kisker et al., 2001). Tests of this mode of administration in bulky disease are currently under way in two countries.

Single agent antiangiogenic therapy may not be the best treatment option for most patients. Antiangiogenic therapy may need to be individually tailored to the patient’s type and stage of cancer, as shown by Bergers et al. (1999). In a mouse model of pancreatic islet cell carcinogenesis, four different angiogenesis inhibitors produced different efficacy profiles. These trials were aimed at prevention of the angiogenesis switch in premalignant lesions, by intervention at the stage of the rapid expansion of small tumors, or by inducing regression of large end-stage cancer (Bergers et al., 1999). The authors concluded that different antiangiogenic drugs proved to be most efficacious when targeted to specific stages of cancer.

Another paradigm that is emerging is the use of combination therapy for the treatment of cancer. Combination therapy with two antiangiogenics (Browder et al., 2000; Klement et al., 2000; Yokoyama et al., 2000a) or combination with radiation therapy (Hanna et al., 2000; Mauceri et al., 1998) or immunotherapy (Davidoff et al., 2001) has been shown to be better than either single therapy. Also, sequential administration of chemotherapy and endostatin was promising for treating bulky non-Hodgin’s lymphoma in a nonobese diabetic/SCID mouse model (Bertolini et al., 2000). More research will be needed to determine doses, scheduling of therapy, and other parameters.

VII. CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Since the discovery of endostatin 5 years ago, many laboratories have contributed to the understanding of endostatin’s biology. The fascinating role for this protein in many

vertebrate systems, especially in developmental biology, is thus far unique among antiangiogenic molecules. Many basic questions remain, however, about the mechanisms of endostatin action. One of the problems in reconciling the many reported in vitro effects is the lack of an identified high-affinity receptor for endostatin. In addition, the relevance of in vitro effects of endostatin to its in vivo effects remains uncertain. The in vitro doses that inhibit proliferation or cause apoptosis are in the μg/mL levels, whereas those required to block cell migration are in the ng/mL range. The picomolar K_d's reported for endostatin's binding to glypican can explain the antimigratory effect of endostatin. It is worth pointing out, however, that this affinity was generated for AP-tagged endostatin. It is well-known that the AP-moiety oligomerizes, and it is possible that this oligomerization potential of AP-ES could have led to lower K_d's. This can be answered by coupling mutant AP that lacks the oligomerization potential to endostatin and testing the binding affinity of the mutant AP-tagged endostatin in endothelial cells.

Genetic ablation of collagen XVIII resulted in normal vasculature everywhere except in the developing eye (Fukai et al., 2002). The growth of tumors in this knockout mouse, however, has not yet been tested. Collagen XVIII is a minor component of the basement membrane of blood vessels. Why, then are high doses or endostatin required for in vivo antitumor effects? B. Olsen (Harvard Medical School) has proposed a dominant negative effect for endostatin's action (i.e., endostatin might inhibit the assembly of collagen in the basement membrane). This, in turn, prevents endothelial cell migration and proliferation, and promotes apoptosis. The cellular localization of exogenous administered endostatin in a tumor-bearing animal may shed light on this hypothesis.

One explanation for the apparently wide-ranging effects of endostatin on neuronal and renal cells might be the widespread expression of its putative receptor with cell specificity conferred by specific downstream signaling pathways. Our research suggests that endostatin might interact with glypican through its sulfated glycan residues. Because proteoglycans (glypicans) show sugar specificity in glycosylation and in sulfation and amidation of those saccharide moieties, it is possible that endostatin's specific effects could be dictated by the glypican structure on endothelial cells. The simpler hypothesis of the presence or absence of the high-affinity receptor may also explain endostatin's specificity. Integrins reported to bind to endostatin (Rehn et al., 2001) might also be a candidate for specificity because different combinations of the α- and β-integrins are present on different cells.

Additional experiments are needed to define the range of endostatin activity and its mechanism of action. The reward may be a novel approach to the treatment of human cancer, and perhaps, other angiogenesis dependent conditions as well.

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TABLE 1

Angiogenesis Inhibitors Generated as Cleaved Fragments from Larger Precursors

Inhibitors	Larger precursors	References
Angiostatin (Kringle1–3, 4)	Plasminogen	(O'Reilly, 1997)
Antithrombin	AaAT	(O'Reilly et al., 1999)
Arresten	Type IV collagen (α1)	(Colorado et al., 2000)
Canstatin	Type IV collagen (α2)	(Kamphaus et al., 2000)
Endostatin	Type XVIII collagen	(O'Reilly et al., 1997)
Heparin-binding fragment	Fibronectin	(Homandberg et al., 1985)
Kringle 5	Plasminogen	(Cao et al., 1997)
Kringle 1–5	Plasminogen	(Cao et al., 1999)
PEX	MMP-2	(Brooks et al., 1998)
PF-4, truncated fragment	Platelet factor-4	(Maione et al., 1990)
Prolactin, 16-kDa fragment	Prolactin	(Clapp et al., 1993)
Restin	Type XV collagen	(Ramchandran et al., 1999)
TSP-1	Thrombospondin	(Good et al., 1990)
Tumstatin	Type IV collagen (α3)	(Maeshima et al., 2000)
Vasostatin	Calreticulin	(Pike et al., 1998)
Vastatin	Type VIII collagen	(Xu et al., 2001)

TABLE 2

A List of Tumors Susceptible to Endostatin's Action In Vivo

Tumor	Source of endostatin	Dose	Reference
786-0 renal clear cell	Murine soluble yeast	10 mg/kg/day	(Dhanabal et al., 1999a)
RC-9, renal cell carcinoma	Human soluble mammalian	10–250 μg/kg/day	(Yamaguchi et al., 1999)
MDA-MB-435 breast orthotopic	Liposome-murine	2.9 μg of plasmid/mouse	(Chen et al., 1999)
B16BL6 melanoma	Human soluble yeast	1.5 mg/kg/day	(Sim et al., 1999)
MA148 ovarian carcinoma	Murine soluble yeast	20 mg/kg/day + angiostatin same dose	(Yokoyama et al., 2000a)
MC38 adenocarcinoma	Adenovirus murine endostatin gene	1×10^9 pfu	(Feldman et al., 2000a)
DMBA induced primary	Rat insoluble endostatin	20 mg/kg/day	(Perletti et al., 2000)
BT4C glioma	Alginate-encapsulated endostatin	0.2 μg/mL per capsule	(Read et al., 2001b)
U-87MG glioma	Alginate-encapsulated human endostatin	150.8 ng/mL	(Joki et al., 2001)