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TWO ENDOGENOUS ANTIANGIOGENIC INHIBITORS, ENDOSTATIN AND ANGIOSTATIN, DEMONSTRATE BIPHASIC CURVES IN THEIR ANTITUMOR PROFILES

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□ Angiogenesis refers to growth of blood vessels from pre-existing ones. In 1971, Folkman proposed that by choking off the blood supply to tumors, they are starved, leading to their demise. A few years ago, the monoclonal antibody Avastin became the first antiangiogenic biological approved by FDA, for treatment of cancer patients. Two other antiangiogenic endogenous protein fragments were isolated in Folkman's laboratory more than a decade ago. Here, we present a short review of data demonstrating that angiostatin and endostatin display a biphasic antitumor dose-response. This behavior is common among a large number of antiangiogenic agents and the reduced effectiveness of antiangiogenic agents at high dose rates may be due to suppression of growth of new vessels carrying the agent into the critical region around the tumor.

Keywords: Angiogenesis, Angiostatin, Endostatin, Biphasic

INTRODUCTION

In 1990, it was reported that thromspondin, an extracellular matrix protein, displayed antiangiogenic properties (Good *et al.* 1990). This finding prompted Judah Folkman to initiate a wide search for circulating endogenous antiangiogenic protein fragments, which presumably regulate angiogenesis in higher organism. Towards achieving this goal, angiostatin was discovered in his laboratory from serum and urine of Lewis-Lung Carcinoma (LLC)-bearing mice (O'Reilly *et al.* 1994). Angiostatin was found to be a degradation product of plasminogen, a major circulating constituent in blood, which contains five kringle domains. Only kringles 1-3 were found to be present in angiostatin (Fig. 1). Apparently,

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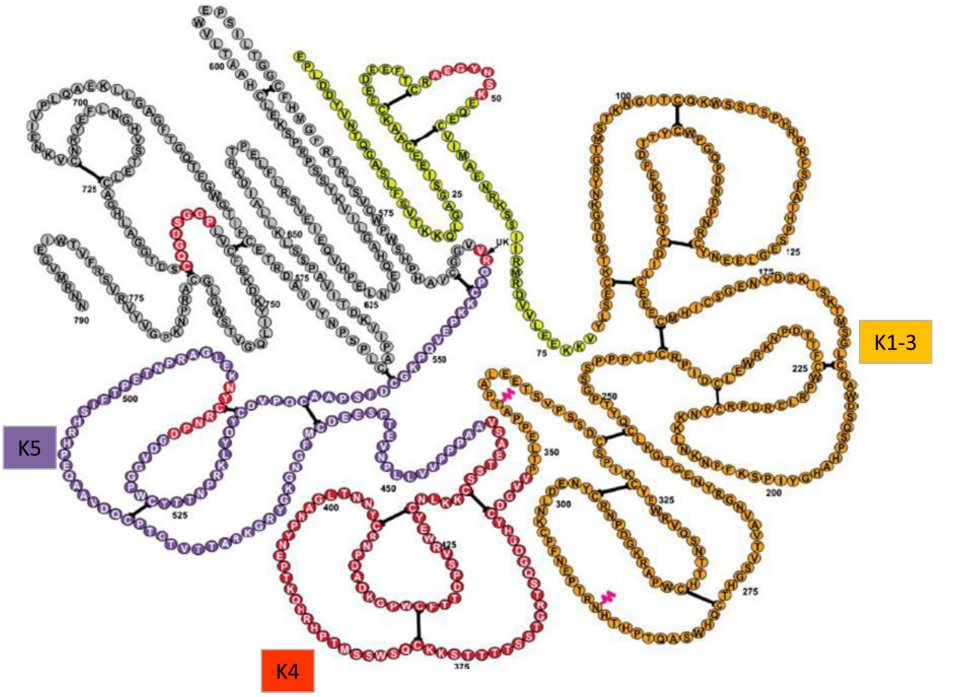

FIGURE 1. The full sequence of human plasminogen containing 5 kringle domains. The N-terminus of angiostatin resides at amino acid 78 (valine). The C-terminus of original angiostatin was not determined, however, based on its molecular size was estimated to be K1-3. The recombinant angiostatin consists of K1-4.

any of the five kringles alone or in combination is capable of exhibiting antiangiogenic activity (Lee *et al.* 2009).

The second endogenous antiangiogenic protein fragment discovered in Folkman's laboratory was endostatin (O'Reilly *et al.* 1997). It is a 20-kDa C-terminal globular domain of collagen 18, first isolated from a hemangioendothelioma cell line for its ability to inhibit the proliferation of capillary endothelial cells. It is derived from a larger precursor molecule called NC-1. Many years ago, in collaboration with the late Don Wiley, we published the crystal structure of endostatin (Fig. 2). It consists mainly of β- structures and forms a dimer through its N-terminus. Each molecule of endostatin binds an atom of zinc through the histidines located at its N-terminus. Most of the antitumor and anti-permeability activities of endostatin can be mimicked by a 25 amino acid peptide responsible for dimerization and zinc binding properties of endostatin (Ding *et al.* 1998, Tjin *et al.* 2005).

Approximately, ten years ago, recombinant endostatin expressed in yeast, was introduced into clinical trial. However, the trial was terminated in phase 1-2, mainly due to the fact that there was not sufficient recombinant protein to continue the trial as a result of poor pharmacokinetics

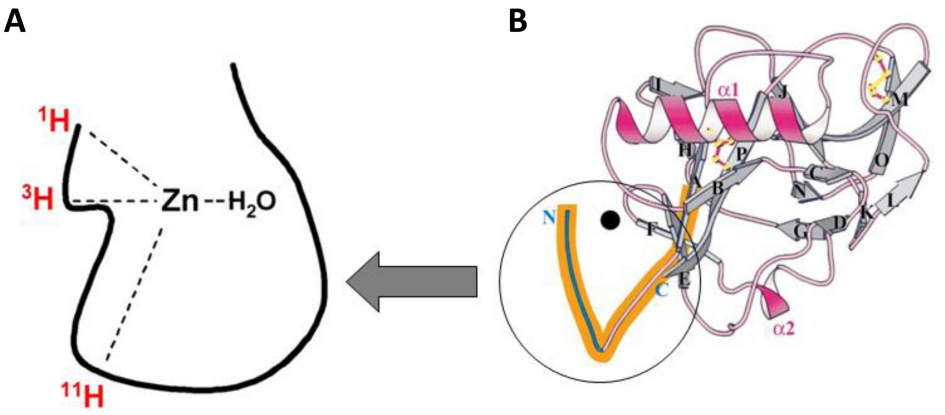

FIGURE 2. Crystal structure of endostatin. The black dot represents zinc atom *(B)*. The orange region corresponds to 25 amino acid peptide at the C-terminus of endostatin (A), which mimics the antitumor activity of the protein. This peptide contains three histidines responsible for zinc binding of endostatin.

of endostatin observed in circulation (Lee *et al.* 2008). The half-life of the clinical grade of endostatin in circulation is only 1-2 hours. In contrast, the majority of biologicals, including monoclonal antibodies, approved for treatment of patients have much longer half-life due to the presence of a Fc domain of IgG, which increases the half-life to weeks instead of hours(Lee *et al.* 2008). In order to address this problem, we engineered a recombinant Fc-endostatin (Fig. 3) that displays a half-life of longer than a week, which is similar to bevacizumab (Avastin, a neutralizing monoclonal antibody directed to VEGF) and VEGF-Trap (directed to the two receptors of VEGF). Bevacizumab in combination with chemotherapy has been approved for use in metastatic colorectal cancer, lung cancer, breast cancer and metastatic renal cancer and soon will likely get approved for the treatment of glioblastoma. VEGF-Trap is in the final phase of several clinical trials.

MECHANISM OF ACTIONS OF ENDOSTATIN AND ANGIOSTATIN

A number of diverse mechanisms have been proposed for endostatin antitumor activity. Among these mechanisms are (i) inhibition of phosphorylation of focal adhesion kinase via binding to integrin α5β1, (ii) interactions with cell surface implicating cell surface glypicans as receptor for endostatin, (iii)blockage of VEGF signaling, (iv) inhibition of wnt-signaling, (v) binding and inactivation of metalloproteinases (Folkman 2006, Abdollahi 2005).

A similar situation exists with respect to the mechanism of angiostatin. Annexin, angiomotin, integrin αvβ3, and c-met have been identified as some of the prominent candidates on the cell surface for binding angiostatin (Wahl *et al.* 2005). ATP synthase has been reported to be a surface-

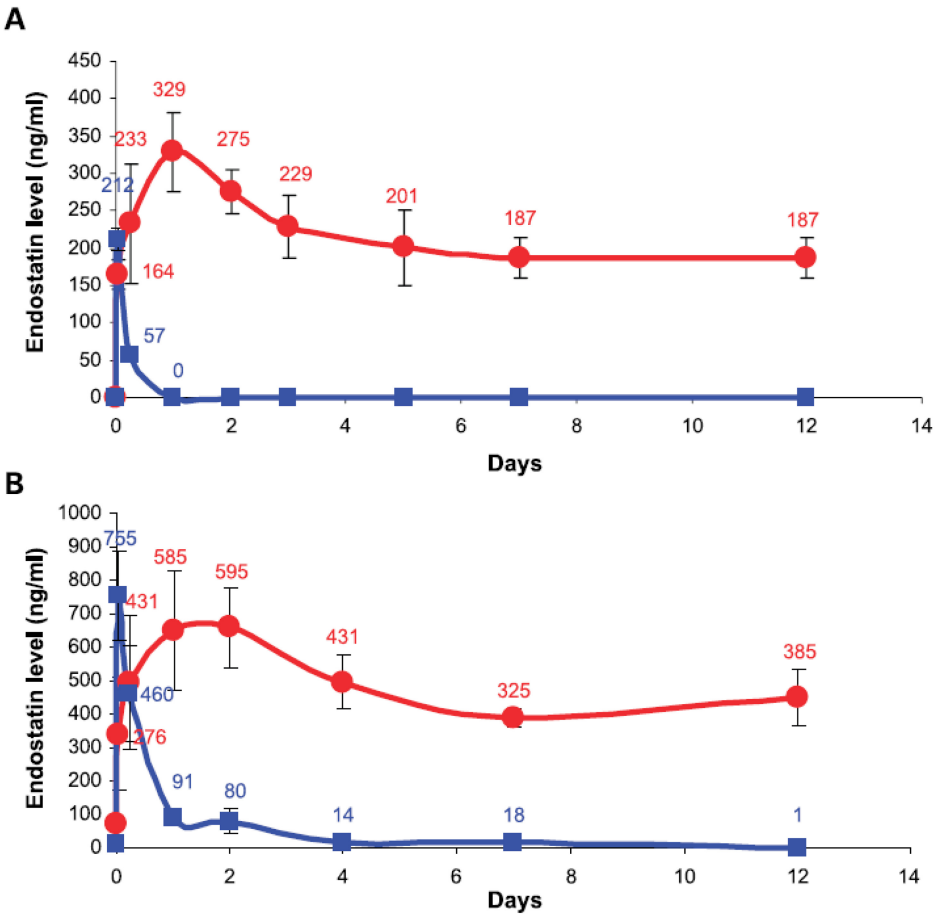

FIGURE 3. Pharmacokinetics of endostatin and Fc-endostatin in mice. Endostatin (100 μg) was injected s.c. into C57Bl/6J mice and concentrations of the circulating protein were monitored by ELISA (CytImmune Sciences, Rockville, MD). A) hFc-endostatin (closed circles) and human endostatin (closed squares). B) mFc-endostatin (closed circles) and mouse endostatin (closed squares). The measured concentrations of mouse endostatin were corrected for baseline endostatin (60 ng/mL).

binding receptor on endothelial cells that selectively binds angiostatin but not plasminogen (Moser *et al.* 1999). Recently, we have demonstrated that angiostatin targets the Krebs cycle in mitochondria (Lee *et al.* 2009). In addition to its presence on the cell surface, ATP synthase is a component of the inner membrane of mitochondria and plays an important role in mediating angiostatin function in mitochondria (Lee *et al.* 2009).

U-SHAPED CURVE BEHAVIOR OF ANTITUMOR PROTEINS

A striking feature of these two antiangiogenic reagents was their demonstration of biphasic dose-response characteristic (Lee *et al.* 2008,

Celik *et al.* 2005, Tjin *et al.* 2006). The optimum antitumor activity was obtained within a narrow range of protein concentration applied to tumor-bearing mice. Below and above this concentration, antitumor activity showed a decrease of activity. This situation is not unique to endostatin and angiostatin. Other proteins that regulate angiogenesis have been reported to show similar biphasic curves of antitumor efficacy, such as IFN-α (Slaton 1999), rosiglitazone (Panigrahy *et al.* 2002) and thrombospondin (Motegi *et al.* 2002).

In our study of Fc-endostatin in mice, we determined that maximum antitumor activity was achieved by administration of approximately 0.7 mg/kg/day. We have compared Fc-endostatin with clinical endostatin in the tumor models ASPC-1 and BxPC-3 (Fig. 4). Maximum antitumor activity was achieved with Fc-endostatin at 0.67 mg/kg/day. In contrast, maximum antitumor activity for endostatin lacking the Fc-fragment was achieved at 100 mg/kg/day for BxPC-3 and 500 mg/kg/day for ASPC-1 (Celik *et al.* 2005). Thus, the optimum antitumor dose for Fc-endostatin is 150- to 700-fold lower than the optimum antitumor dose for endostatin that lacks the Fc-fusion domain.

Similarly, angiostatin displays a biphasic profile as endostatin (Fig. 5) (unpublished data).

WHAT IS THE BASIS OF BIPHASIC BEHAVIOR OF ENDOSTATIN AND ANGIOSTATIN

In order to explain the U-shaped curves observed here, we first hypothesized that endostatin might become oligomeric at high concentration of the protein and consequently would be interfering with its binding to the receptor. However, at least two pieces of data argue against this proposition. First, endostatin is derived from trimeric NC-1, which is the physiological ligand. Our preliminary results demonstrate that NC-1 has a higher antitumor activity than endostatin (unpublished data). The second piece of evidence is the fact that U-shaped curve is observed in a large number of antitumor biologicals, which very likely have different mechanisms of action.

A more satisfactory explanation is that there are two targets for these proteins with separate S-shaped curves. The effective target has a much lower NOEL (No Observed Effective Level) but an ED50 similar to the second target (Conolly and Lutz 2004). The second target is usually a transport protein or regulator of membrane opening such that when it shuts down, the drug does not get to the first target. Ironically, in the case of drugs that inhibit angiogenesis, very high concentration might inhibit or reverse the development of capillaries that bring the drug to the critical zone immediately in contact with the tumor. Thus, very high dose rates may actually suppress the amount of active drug reaching the tumor to counteract the effects of the angiogenic inhibitor.

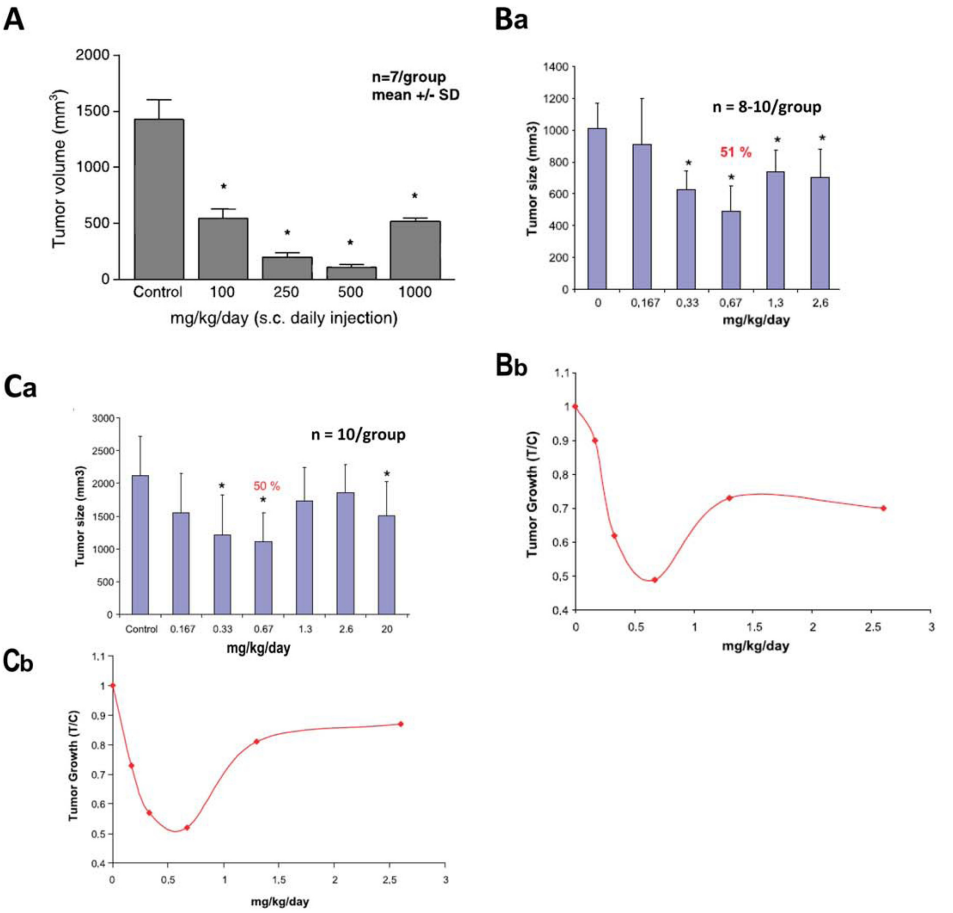

FIGURE 4. Biphasic anti-tumor activity of endostatin. A) Treatment of SCID mice, bearing human ASPC-1 pancreatic tumors with daily injection of clinical grade endostatin (Celik *et al.* 2005) (Reprinted with permission of publisher and authors). Mean (+ SD) tumor volume after a 16-day treatment with different dosages of endostatin. B_a) Similar to (A), except Fc-endostatin was employed. Note the difference of endostatin doses required to achieve antitumor effects with clinical grade endostatin and Fc-endostatin. C_a) Treatment of SCID mice bearing human melanoma A2058 tumor cells with Fc-endostatin. **P <0.001.*
B_b and C_b refer to tumor inhibition percentages (T/C) for the same data in Fig. B_a and C_a respectively (Lee *et al.* 2008). Number of mice in each group is designated by "n".

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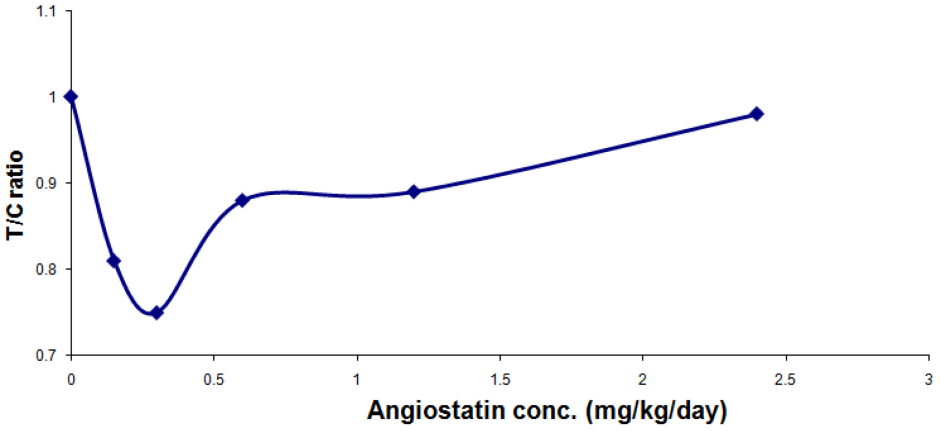

FIGURE 5. Biphasic anti-tumor activity of angiostatin. Mouse Fc-angiostatin was injected in C57Bl/6J mice bearing Lewis-Lung Carcinoma (LLC). This is an aggressive tumor model and a shorter period of time allows one to obtain significant tumor size differences as a result of treatment.

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