A Recombinant Human Angiostatin Protein Inhibits Experimental Primary and Metastatic Cancer

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ABSTRACT

Endogenous murine angiostatin, identified as an internal fragment of plasminogen, blocks neovascularization and growth of experimental primary and metastatic tumors in vivo. A recombinant protein comprising kringles 1–4 of human plasminogen (amino acids 93–470) expressed in Pichia pastoris had physical properties (molecular size, binding to lysine, reactivity with antibody to kringle 1–3) that mimicked native angiostatin. This recombinant Angiostatin protein inhibited the proliferation of bovine capillary endothelial cells in vitro. Systemic administration of recombinant Angiostatin protein at doses of 1.5 mg/kg suppressed the growth of Lewis lung carcinoma-low metastatic phenotype metastases in C57BL/6 mice by greater than 90%; administration of the recombinant protein at doses of 100 mg/kg also suppressed the growth of primary Lewis lung carcinoma-low metastatic phenotype tumors. These findings demonstrate unambiguously that the antiangiogenic and antitumor activity of endogenous angiostatin resides within kringles 1–4 of plasminogen.

INTRODUCTION

Many studies demonstrate the essential role of angiogenesis, the process of new blood vessel formation, in the aggressive growth of solid tumors and their metastases (1–4). In certain experimental and naturally occurring neoplastic diseases, excision of the primary tumor results, within weeks, in the fulminating growth of previously dormant metastases. These observations stimulated an interesting hypothesis (reviewed in Refs. 5–7) that ascribed the inhibition of tumor growth to tumor mass. O'Reilly and colleagues illustrated at least one mechanism by which this inhibition might be achieved: systemic control of distal tumor angiogenesis by a byproduct of primary tumor growth (8). They showed that a primary LLC-LM2 tumor, although capable of stimulating angiogenesis in its own vascular bed, can inhibit angiogenesis in the vascular beds of its metastases. Thus, metastases are seeded in the lungs of mice with a primary LLC-LM tumor, but these metastases remain avascular and fail to grow beyond microscopic size unless the primary tumor is removed. An endogenous Mr, 38,000 protein purified from serum and urine of mice with primary LLC-LM tumor, named Angiostatin protein, reproduced in mice from which tumors had been excised the same phenomenon that was seen in mice with intact tumors: control of metastatic tumor growth in the lungs (8). NH2-terminal sequencing of the Mr, 38,000 endogenous mouse Angiostatin protein identified it as a fragment of mouse plasminogen starting with amino acid 98 (8). [The sequence numbering in this report starts with the first methionine as amino acid 1. Traditional numbering of plasminogen starts with the first amino acid of the mature protein, which is Gly19 in the case of mouse plasminogen (9).]

Microsequencing of portions of the protein showed that it encompassed at least the first three of the five highly homologous, looped, multiple disulfide-bonded domains, each of approximately 80 amino acids, called kringles (10). Several lines of evidence reported by O'Reilly et al. (8) strongly suggest that the Mr 38,000 endogenous Angiostatin protein is an internal fragment of plasminogen: (a) protein sequence information obtained from the murine endogenous protein fragments (23% of total protein) showed identity with mouse plasminogen; (b) limited elastase digestion of human plasminogen results in a protein, native human Angiostatin protein, that inhibits bFGF stimulated proliferation of BCE cells in vitro and angiogenesis in vivo in chick chorioallantoic assays, whereas intact human plasminogen is inactive in these assays. The same native human Angiostatin protein administered systemically, but not intact human plasminogen, inhibits angiogenesis and the growth of lung metastases in the mouse LLC-LM model; and (c) all antimetastatic and antiendothelial activity in the urine of tumor-bearing mice is removed by immunoaffinity chromatography using an antibody reactive against kringle 1–3 of human plasminogen. A recent study also shows that native human Angiostatin protein induces and sustains dormancy of human primary tumors in mice (11).

On the basis of the NH2-terminal sequence and the predicted molecular size of the endogenous murine and native human Angiostatin proteins, we selected the first four kringle regions of human plasminogen to express in Pichia pastoris. We report that the expressed recombinant protein is folded so that it reacts with a conformation-dependent antibody against kringle 1–3 of plasminogen, and the recombinant protein exhibited the chemical and in vitro biological properties of native human Angiostatin protein. More important, this recombinant Angiostatin protein suppressed the growth of LLC-LM primary tumors and metastases when administered systemically. A recombinant Angiostatin protein with these activities may provide an opportunity to control tumor development in a clinical setting through the therapeutic strategy of angiogenesis inhibition.

MATERIALS AND METHODS

Cloning the Gene Encoding Human Plasminogen Kringles 1–4 into P. pastoris. Sequences encoding Angiostatin protein were amplified by PCR using Vent polymerase (New England Biolabs); primers 154 (5'-ATCGCTC-GACCGTTAATTTGAAAGAAGTGAAT-3') and 151 (5'-ATCGGAATTCAAGCAGGACAAACCAGGCGG-3'), containing linkers with XhoI and EcoRI restriction sites, respectively; and the plasmid pTrCHis/HAs as template. This plasmid contained sequences encoding amino acids 93–470 of human plasminogen (Ref. 12; amino acid 1 is the first methionine) for cloning into the XhoI/EcoRI site of pHIL-S1 (Invitrogen, San Diego, CA) expression vector using P. pastoris native secretion signal, PHO 1. The product of the amplification was gel purified and ligated with the appropriate enzymes and then again purified using Gene-Clean (Bio 101, La Jolla, CA). The gene fragment was ligated into pHIL-S1. Resultant clones were selected and plasmid preparations of clones were obtained and linearized at the BygII site to generate His13 Mut1 and His13 Mut1 recombinant strains when transformed into P. pastoris host strain GS115. Integration was confirmed by PCR.

Both His13 Mut1 and His13 Mut1 recombinants were induced with methanol and screened for high expression of recombinant Angiostatin protein using Coomassie-stained SDS-PAGE gels and immunoblots with the murine mono-
clonal against kringles 1–3 (Enzyme Research Laboratories, Inc., South Bend, IN). From these, a GS115-transformed P. pastoris clone, pHIL-S1/HA18, was selected and phenotypically characterized as His<sup>+</sup> Mut<sup>E</sup> by patching on minimal methanol and minimal dextrose agar plates.

Expression of Clone pHIL-S1/HA18. Expression of recombinant Angiostatin protein by pHIL-S1/HA18 was typical for a His<sup>+</sup> Mut<sup>E</sup> clone. For induction in baffled shake flasks, 1 liter of A<sub>600 nm</sub> 4–5 cells were cultured in 150 ml of buffered methanol complex medium containing 1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% yeast nitrogen base with ammonium sulfate, 0.00004% biotin, and 0.5% methanol in a 1-liter baffled flask. Cells were rotated at a constant speed of 250 rpm at 30°C. Absolute methanol was delivered in batches at 24-h intervals to a final concentration of 0.5%. After 120 h, cells were centrifuged at 5000 rpm for 10 min, and culture broth was stored at −70°C until used.

Purification of Recombinant Angiostatin from Fermentation Broth by Lysine-Sepharose Chromatography. All purification procedures were carried out at 4°C. Crude fermentation broth (200 ml) containing recombinant Angiostatin protein was clarified by centrifugation at 14,000 × g, concentrated, and buffer exchanged against 50 mM phosphate buffer, pH 7.5, by Centriprep 30 (Amicon; a M<sub>W</sub> 30,000 cutoff membrane) to approximately 25% of the original volume. Lysine-Sepharose 4B (Pharmacia) was used as an affinity substrate. A 48 × 100-mm lysine-Sepharose column was packed and equilibrated with 50 mM phosphate buffer, pH 7.5. The sample was pumped onto the column at a flow rate of 1.5 ml/min, and the column was washed with 1.5 column volumes of 50 mM phosphate, pH 7.5, at a flow rate of 3 ml/min. The column was then washed with 1.5 column volumes of PBS, pH 7.4, at a flow rate of 3 ml/min. Recombinant Angiostatin protein was eluted with 0.2 M e-aminocaproic acid, pH 7.4, at a flow rate of 3 ml/min. Fractions containing significant absorbance were pooled and dialyzed for 24–48 h against deionized water and lyophilized. A typical recovery from 100 mg of total protein load was 10 mg recombinant Angiostatin protein. Columns were regenerated using 5 column volumes of 50 mM phosphate/1 M NaCl, pH 7.5.

BCE Cell Proliferation Assay. BCE cells were obtained as described previously (13). The cells were maintained in DMEM containing 3 ng/ml of recombinant human bFGF (Scios Nova, Mountainview, CA), supplemented with 10% heat-inactivated bovine calf serum, 100 units/ml penicillin, 100 mg/ml streptomycin, and 0.25 mg/ml fungizone (BioWhittaker) in 75-cm<sup>2</sup> cell-culture flasks. The assay was performed as described previously (8). To ensure that any inhibition seen was not due to the test material detaching the BCE cells from the plate, all wells of the assay were studied under an inverted microscope for evidence of cell detachment prior to cell counting.

Tumor Cell Line. The low metastatic LLC-LM was maintained by repeated in vivo passage by selecting for the appropriate phenotype (i.e., mice with tumors that suppressed growth of their metastases completely and had no visible surface lung metastases were used for LLC-LM passage). Tumor cells (1 × 10<sup>6</sup>) were implanted into the s.c. tissue of the mice in the proximal midline of the dorsal surface in 0.1 ml of saline as described previously (8).

Inhibition of LLC-LM Metastases in Mice. Six to 8-week-old male C57Bl/6J mice (The Rosco Jackson Laboratory) were implanted with 1 × 10<sup>6</sup> LLC cells (LLC-LM) as described previously (8). When tumors reached 1500 mm<sup>2</sup> in size, approximately 14 days after implant, they were surgically removed using aseptic techniques as described previously (8). Tumors were left intact in one group of mice to serve as an endogenous Angiostatin protein control. Some of the animals in the tumor-resected group received a first dose (3 mg/kg by the s.c. route) of recombinant or native Angiostatin protein immediately after surgery, followed by daily doses of 1.5 mg/kg for 14 days. A control group of mice received an equal volume of PBS every day for 14 days following surgery. All mice were sacrificed 14 days after primary tumor removal (28 days after tumor implantation), lungs were removed and weighed, and surface metastases were counted with a stereomicroscope at ×4 magnification.

Inhibition of Primary LLC-LM Tumors in Mice. Primary LLC tumors were implanted as described (8). After 5–7 days, when tumors were palpable and were 3–4 mm in diameter, groups of five mice were treated with either 100 mg/kg recombinant Angiostatin protein or PBS each day. Treatment was terminated after day 3 due to limited amounts of purified recombinant Angiostatin protein. The size of the tumors in all groups were measured at the same time every day with dial calipers.

Histology. Lungs were fixed in Carnoy’s fixative for 4 h and then in absolute ethanol before being embedded in paraffin according to standard histological procedures. Sections were prepared and immunostained with antibodies against von Willebrand factor using a standard kit (Biomedica, Inc., Foster City, CA) performed according to the manufacturer’s instructions. Quantitation of anti-von Willebrand staining (microvessels per field) were performed under ×400 magnification of a standard light microscope. The number of anti-von Willebrand-stained cells per number of tumor cells was scored. A total of 5000 tumor cells were counted.

RESULTS

Characteristics of Recombinant Human Angiostatin Protein. The gene fragment encoding kringles 1–4 of human plasminogen, which contains a total of 26 cysteines, was expressed in the methylothropic yeast P. pastoris using the native PHO 1 secretory signal. The recombinant Angiostatin protein bound to lysine Sepharose and could be specifically eluted by e-aminocaproic acid. This suggested that fully functional lysine-binding kringle(s), a physical property of correctly folded kringles 1 and 4 of plasminogen (10, 14, 15), could be purified from the culture broth of P. pastoris by techniques that did not require refolding (Fig. 1). Both recombinant Angiostatin protein from P. pastoris and native human Angiostatin protein produced by elastase cleavage of plasminogen were recognized by a conformation-dependent monoclonal antibody against kringles 1–3 (Enzyme Research Laboratories, Inc., South Bend, IN; Fig. 1B). This

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**Fig. 1.** Lysine-binding characteristics and immunoreactivity of recombinant Angiostatin protein. A, Coomassie-stained gel (40 μl/ lane) Lane 1, broth from shake flasks of induced cultures showing recombinant Angiostatin protein at about M<sub>W</sub> 50,000 and a few other proteins. Broth from induced cultures was diluted 1:1 with buffer and directly loaded onto lysine-Sepharose. Lane 2, unbound fraction that passed through the lysine column. All recombinant Angiostatin protein bound to the lysine column. Lane 3, specific elution with 0.2 M amino caproic acid showed that recombinant Angiostatin protein bound to lysine and could be purified to homogeneity in a single step using a lysine-Sepharose column and an immunoblot (20 μl/lane) of a gel similar to the Coomassie gel (B). Recombinant Angiostatin protein is recognized by a conformationally dependent monoclonal antibody raised against kringles 1–3 (16).
Antibody does not recognize reduced forms of plasminogen (16) or Angiostatin protein (data not shown).

Recombinant Angiostatin protein shared several important characteristics with plasminogen. In nature, two glycosylation forms of plasminogen exist in each individual: type I (−33%) is glycosylated at Asn289 and at Thr346; type II (−67%) is only glycosylated at Thr346 (Refs. 17 and 18; the amino acid numbering here is in accordance with the traditional method, in which the first amino acid is that of the mature protein). Recombinant Angiostatin protein was a doublet of Mr 49,000 and 46,500 by mass spectrometry and ran at about Mr 49,000 (Figs. 1 and 3A). Silver staining of the purified recombinant Angiostatin protein showed the doublet and no other obvious components (Fig. 3A). Silver staining of the purified recombinant Angiostatin protein showed the doublet and no other obvious components (Fig. 3A). It is noted that this preparation of recombinant Angiostatin protein consisting of the doublet was used in all in vitro and in vivo assays reported in this paper. Proteins expressed in P. pastoris are posttranslationally modified; most proteins are modified with an N-linked glycosylation of the high-mannose type, and virtually none are modified by O-linked glycosylation (19). To evaluate glycosylation in recombinant Angiostatin protein, we digested the recombinant protein with endoglycosidase H, which is specific for high-mannose structures. This caused the Mr 51,000 band to migrate identically with the band at Mr 49,000 (Fig. 2). O-glycanase digestion with prior neuraminidase treatment to remove sialic acid residues did not change the pattern of migration of the doublet (data not shown). NH2-terminal amino acid sequencing of the doublet showed identical sequences starting with amino acid 93, as was cloned in the construct. These results suggest that P. pastoris expressed Angiostatin protein in two forms: (a) a form with an N-linked complex carbohydrate chain; and (b) an unglycosylated form.

Inhibition of BCE Cells in Vitro. To determine whether recombinant Angiostatin protein had antiangiogenic activity, we cultured BCE cells in the presence of bFGF and asked whether the addition of purified recombinant Angiostatin protein inhibited proliferation. Purified P. pastoris-expressed Angiostatin protein (Fig. 3A) inhibited the bFGF-induced proliferation of BCE cells in vitro (Fig. 3B) in a dose-dependent manner (Fig. 3C). Similar inhibitory effects on BCE cells were seen when native human Angiostatin protein was used in the same assay (Fig. 3, B and C). This assay was performed in triplicate eight times. In these eight experiments, the range of inhibition of BCE cells was 60–80% for both recombinant Angiostatin protein and native human Angiostatin protein used separately at 2 µg/ml (data not shown). At 2 and 1 µg/ml, the proliferation of BCE cells treated with either recombinant or native Angiostatin protein was significantly different from the proliferation of untreated BCE cells (P < 0.005). The proliferation of BCE cells treated with either recombinant or native Angiostatin protein at 0.5 µg/ml was significantly different from the proliferation of untreated BCE cells (P < 0.05). Half-maximal inhibition was seen with both recombinant Angiostatin protein and with native human Angiostatin protein concentrations between 0.5–1 µg/ml. Neither protein inhibited the growth of LLC-LM tumor cells (data not shown). There was also no evidence of BCE cell detachment from the assay plates even at the highest concentration of Angiostatin protein tested, prior to cell counting.

Inhibition of Metastatic Tumor Growth in Vivo. Recombinant Angiostatin protein administered systemically at 30 µg/mouse/day (equivalent to 1.5 mg/kg/day) inhibited the growth of metastatic lung...
Systemic treatment with recombinant Angiostatin protein inhibited the growth of metastases after removal of the primary tumor. Number of metastases (A) and the lung weights (B) of mice treated daily with saline or recombinant Angiostatin protein or plasminogen-derived Angiostatin protein. Columns, mean; bars, SE.

H&E-stained sections of lungs of all mice in the study demonstrated metastases in all groups, but metastases in lungs of mice treated with recombinant Angiostatin protein or with native human Angiostatin protein were small and isolated (data not shown). Lung tissue sections from all groups of mice were immunohistochemically stained with antibodies against von Willebrand factor (an endothelial cell-specific marker). Within the lung metastases, those from mice treated with recombinant Angiostatin protein showed anti-von Willebrand factor staining of preexisting lung vessels with very few localized areas of staining within the metastases, which is indicative of little neovascularization (Fig. 6A). In comparison, for a similar cross-sectional area of tumor tissue, the lung metastases from control mice treated with saline were densely stained with this specific endothelial cell marker, which indicates highly vascularized tumor tissue (Fig. 6B). The results of reactivity with anti-von Willebrand factor suggest that recombinant Angiostatin protein directly inhibited angiogenesis. Within the lung metastases, scoring of the number of anti-von Willebrand factor-stained cells per 5000 cells randomly selected from cross-sectional areas showed that 11.1% of the cells were stained in lung metastases of mice treated with saline compared to 2.3% of the cells in lung metastases of mice treated with recombinant Angiostatin protein. The in vivo studies detailed in Figs. 4–6 were repeated. In this second study, mice with primary tumors resected (n = 4) were treated with daily doses of 30 µg of recombinant Angiostatin protein. The lungs of these animals weighed 178 ± 10 mg, in contrast to the lungs of mice treated with saline (423 ± 60 mg; P < 0.05). There were small numbers of surface metastases (8 ± 5) on lungs of mice treated with recombinant Angiostatin protein. In contrast, lungs from mice with primary tumors resected that were treated with saline (n = 3) were completely covered with highly vascularized metastases that were too numerous to count.

**Inhibition of Primary Tumor Growth In Vivo.** Recombinant Angiostatin protein administered at 100 mg/kg/day (2 mg/mouse/day) dramatically inhibited the growth of primary LLC in mice (n = 5; Fig. 7). We had enough recombinant material to give 5 doses over 3 days (1 mg/mouse in the a.m., 1 mg/mouse in the p.m.) only. Tumor growth was suppressed as long as Angiostatin protein was administered. In contrast, equivalent doses of plasminogen, BSA, or improperly folded kringle of plasminogen expressed in Escherichia coli did not affect the growth of primary LLC-LM tumors (data not shown).
Effects of Treatment with Recombinant Angiostatin Protein.

Treated and control animals were similar in outward appearance, feeding habits, and behavior at the dose and regimen used in this study. There was no evidence of inflammation or bleeding in any of the mice treated with the recombinant or enzyme-cleaved protein.

DISCUSSION

These findings provide unambiguous evidence that the antiangiogenic activity of endogenous murine and native human angiostatin, as reported by O'Reilly et al. (8, 11), resides within amino acids 93–470 of plasminogen, the kringle 1–4 plasminogen fragment expressed by P. pastoris. In this study, we show that recombinant Angiostatin protein possesses two important physical characteristics of the natural protein: it was recognized by a conformation-dependent antibody raised against kringle 1–3 of human plasminogen and it bound lysine. These properties suggest that the recombinant Angiostatin protein was expressed with a conformation that mimics the native molecule. We also show that recombinant Angiostatin protein inhibited the proliferation of BCE cells stimulated by bFGF in vitro. Importantly, when administered systemically, recombinant Angiostatin protein maintained the otherwise lethal LLC in a suppressed state. In contrast, equivalent doses of plasminogen did not inhibit the growth of LLC-LM metastases (8). The growth of primary LLC-LM tumors was also not affected by equivalent doses of plasminogen, BSA, or improperly folded kringle 4 of plasminogen expressed in E. coli given on the same schedule (data not shown).

It is intriguing that both the recombinant protein and native human plasminogen are produced in a glycosylated and a nonglycosylated form. In the case of human plasminogen, a single transcript from a single gene can produce both forms, the molecular mechanism(s) of differential posttranslational modifications of human plasminogen, as well as those of other kringle-containing proteins of the blood clotting system, such as tissue plasminogen activator (20), are unknown. The importance of glycosylation as it relates to the in vivo activity of Angiostatin protein is still under investigation. Receptor recognition for Angiostatin protein may require both the peptide and the sugar chain, analogous to the binding of endothelial cell P-selectin to its ligand on leukocytes. In this situation, the binding site reacts with the heavily glycosylated mucin domain, but the protein core dictates the specificity of the interaction (21). The requirement for both the sugar chain and the protein backbone of other proteins with repeated cysteine motifs (as seen in the kringle domains of plasminogen, heparan sulfate, and others) in receptor-ligand interactions is now established in a number of systems (22).

The role of glycosylation in the rate of clearance of recombinant Angiostatin protein is also not yet understood. In mice, up to 50% of unglycosylated E. coli-expressed human plasminogen, enzymatically deglycosylated plasminogen, and asialoplasminogen type II is cleared from circulation within 10 min, compared to 45 min for native plasminogen type I (23). In that study, however, clearance of native plasminogen type II was not studied. In mice, endogenous Angiostatin protein had a long calculated half-life in circulation (greater than 2 days), but the native human Angiostatin protein had a considerably shorter calculated half-life of 4 h (8).

What is the source of Angiostatin protein in vivo? Plasminogen is produced by the liver and is maintained at a stable plasma concentration of 1.6 ± 0.2 μM (24). Messenger RNA transcripts for plasminogen or for Angiostatin protein were not detected in murine Lewis lung tumors freshly resected from mice or in LLC cells after up to 4 passages in vitro (25). LLC-LM tumors might, however, produce an enzyme that cleaves plasminogen, either bound or in circulation, to produce Angiostatin protein (8, 25). Alternatively, inflammatory cells infiltrating the primary tumor could produce such an enzyme (25). Macrophages, in particular, produce elastases (26) that may be involved in the production of Angiostatin protein at the primary tumor site.

The interaction of plasminogen, plasminogen activators, and plasminogen activator inhibitors with endothelial cells is remarkably complex (reviewed in Ref. 27). Plasminogen binds directly to cultured human umbilical vein endothelial cells via kringle 5 (28). An endothelial cell receptor has been identified for plasminogen, the calcium- and phospho-

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*Fig. 6. Immunohistochemical analysis of vascularization of lung metastases was performed by staining with polyclonal antibody against endothelial cell-specific von Willebrand factor. A, a typical cross section of a lung metastases in a mouse treated with recombinant Angiostatin protein. Seen in the section are positively stained preexisting lung vessels and very few positively stained endothelial cells. B, a similar cross sectional area of a typical lung metastases from control mice treated with saline. These lung metastases had readily detectable von Willebrand staining indicative of highly vascularized tumor tissue. X400.*

*Manuscript in preparation.*
lipid-binding protein annexin II (29, 30). Annexin II preferentially binds Lys-plasminogen (31) and also tissue plasminogen activator and may promote and localize constitutive plasmin generation on the surface of endothelial cells (30). It is possible that a similar mechanism of Angiostatin protein generation occurs on endothelial cell surfaces.

The inhibitory effect of recombinant Angiostatin protein on endothelial cells in vitro and its inhibition of tumor growth in vivo, combined with its definitive molecular composition and structure, is an important advance in our understanding of the role of this protein on tumor inhibition by tumor mass. The activity of this recombinant protein, which is qualitatively indistinguishable from the protein described by O’Reilly et al. (8), closes the loop between the substantial and impressive circumstantial evidence of the role of endogenous murine or native human Angiostatin protein in this inhibition and the definitive proof of direct activity of recombinant kringles 1–4 of plasminogen, by itself, to inhibit tumor growth. The studies in this report pave the way for testing the clinical utility of a recombinant product with antiangiogenic and antitumor activity in patients with neoplastic diseases dependent upon angiogenesis for progression.

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Fig. 7. Recombinant Angiostatin protein therapy of mice bearing LLC tumors. Data points, mean tumor diameter for five mice in each treatment group, bars, SE. Mice treated with Angiostatin protein received two treatments of 1 mg/mouse on days 1 and 2 and one treatment of 1 mg/mouse on day 3.

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