KY) and APC (C-20; Santa Cruz Biotechnology, Santa Cruz, CA). Slides stained with antibody to Tcf1 (*9*) were immersed in a 6 mM tris, 10 mM EDTA steam bath (pH 8.0) for 20 min. The primary antibody was detected with horseradish peroxidase (HRP)–conjugated rabbit antibody to mouse (anti-mouse) immunoglobulin G (IgG) or HRP-conjugated swine anti-rabbit IgG, or both (DAKO, Copenhagen, Denmark). Diaminobenzidine (DAB; Sigma) was used as substrate.

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16. Transfections in IIA1.6 B cells were carried out as in (*7*), with 1 μg of TRR-luciferase reporter, 2 μg of hTcf4, and 0, 2, 4, or 6 μg of β-catenin–encoding plasmids. pCDNA was used to bring the total amount to 9 μg of plasmid DNA. Fifty nanograms of a cytomegalovirus promoter–Renilla plasmid was added as an internal control to monitor transfection. LS174T cells were transfected by Fugene 6 (Boehringer Mannheim, Mannheim, Germany). TRR-luciferase reporter (0.5 μg) and 0, 1.5, or 4.5 μg of ΔNTcf4-encoding plasmids were used.
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Antiangiogenic Activity of the Cleaved Conformation of the Serpin Antithrombin

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Antithrombin, a member of the serpin family, functions as an inhibitor of thrombin and other enzymes. Cleavage of the carboxyl-terminal loop of antithrombin induces a conformational change in the molecule. Here it is shown that the cleaved conformation of antithrombin has potent antiangiogenic and antitumor activity in mouse models. The latent form of intact antithrombin, which is similar in conformation to the cleaved molecule, also inhibited angiogenesis and tumor growth. These data provide further evidence that the clotting and fibrinolytic pathways are directly involved in the regulation of angiogenesis.

For a carcinoma to expand beyond a prevascular size, it must produce stimulators of angiogenesis in excess of inhibitors (*1*, *2*), and the continued production of the inhibitors provides one mechanism for the inhibition of tumor growth by tumor mass (*3*, *4*). Using murine models, we identified the angiogenesis inhibitors angiostatin (*3*) and endostatin (*4*). To determine if human tumors produce similar inhibitors, we screened small-cell lung cancer cell lines for their ability, when grown on a mouse flank, to inhibit the growth of a comparable implant on the opposite flank. We chose small-cell lung cancer because, clinically, metastases can grow rapidly after treatment of primary disease. One cell line, NCI-H69, inhibited the growth of a secondary implant by 80%. By selective in vivo passage, variants of this line were developed. In one variant, H69i, the inhibition of one tumor by the other was virtually 100% (Fig. 1A). In a second, H69ni, there was no inhibition (Fig. 1B).

Using a 72-hour proliferation assay (*4*), we detected an inhibitor of endothelial cell proliferation in conditioned media of the H69i cell line. The activity was purified (*5*) to apparent homogeneity, eluted at 54 to 56% acetonitrile from a C4 high-performance liquid chromatography (HPLC) column, and was associated with a 58-kD band that migrated as two bands of 53 to 55 kD and 3 to 5 kD under reducing conditions. The inhibitory band initially copurified with one that migrated (reduced) at 58 to 60 kD (Fig. 1C). NH_2-terminal microsequence analysis revealed identity to bovine antithrombin (Fig. 2). Microsequence analysis of both the 53- to 55-kD and the 3- to 5-kD band revealed that the inhibitory protein is cleaved bovine antithrombin (Fig. 2). The cleavage site between Ser^{386} and Thr^{387} has not previously been described. Enzymes that cleave antithrombin include thrombin (Arg^{394}-Ser^{395}) and pancreatic (Val^{388}-Iso^{389}) and human neutrophil elastase (Iso^{391}-Ala^{392}) (*6*, *7*). Conditioned media from the H69ni cells did not substantially inhibit endothelial cell proliferation even when applied to heparin Sepharose with a protocol similar to that described (*8*). These data strongly suggest that the inhibition of angiogenesis by cleaved antithrombin from the H69i cells is in part responsible for the inhibition of tumor growth observed in vivo.

Antithrombin circulates in a quiescent form in which its reactive COOH-terminal loop is not fully exposed and cannot bind target proteases. Heparin induces a stressed conformation of the molecule, exposes the reactive loop (*6*, *9*), and increases thrombin affinity by up to a factor of 100 (*6*). The thrombin-antithrombin complex can slowly dissociate, and the reactive loop of antithrombin is cleaved by the released thrombin (*10*, *11*). Cleaved antithrombin consists of disulfide-bonded A and B chains and does not bind target proteases. Cleavage induces a conformational change to a relaxed (R) form in which the loop irreversibly inserts into the A-beta sheet (*12*). A similar irreversible conformational change of antithrombin to a latent form has been described (*13*, *14*). Mild denaturation of the molecule (*13*) induces a locked conformation characterized by polymers of the latent molecule, and heat treatment with citrate produces a latent monomeric antithrombin (*14*).

Cleaved antithrombin was purified from bovine calf serum (*15*), and human antithrombin was purified from outdated plasma (*15*) and cleaved with pancreatic elastase (*16*). The cleaved antithrombin potently inhibited endothelial cell proliferation induced by bovine fibroblast growth factor (Fig. 3) or by vascular endothelial growth factor (*8*) in a dose-dependent fashion with half-maximal inhibition seen at 50 to 100 ng/ml. The stressed conformation of antithrombin had no substantial effect on capillary endothelial cell proliferation at comparable doses (Fig. 3) but did show marginal inhibition at doses in excess of 5 μg/ml.

To produce the locked conformation (*13*), we incubated antithrombin in 0.9 M guanidine and then performed dialysis. Monomeric latent human antithrombin was produced as described by incubating stressed antithrombin (0.5 mg/ml) in 0.25 M trisodium citrate and 10 mM tris-HCl (pH 7.4) at 60°C for 18 hours (*14*). Both potently inhibited capillary endothelial cell proliferation in a dose-dependent and reversible fashion with half-maximal inhibition observed at 50 to 100 ng/ml (*8*). These data demonstrate that the conformational change that occurs after cleavage of antithrombin confers antiangiogenic activity, and we refer to this conformation as antiangiogenic antithrombin (aaAT).

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To determine if the inhibitory activity was specific for endothelial cells, we tested stressed and cleaved antithrombin on bovine aortic smooth muscle, 3T3 fibroblasts, Madin-Darby canine kidney epithelial cells, human Sloan-Kettering–neuroblastoma-AS (SK-NAS) cells, murine Lewis lung carcinoma, and the H69 cells in vitro (*3*). None of these nonendothelial cell lines were substantially inhibited even at doses up to 100 times as high as those used to inhibit endothelial cells (*8*).

To determine if aaAT could inhibit in vivo angiogenesis, we tested it in the chick chorioallantoic membrane (CAM) assay (*17*). Stressed antithrombin had no effect on angiogenesis and, at doses above 20 μg, caused local hemorrhage. In contrast, cleaved and locked antithrombin potently inhibited angiogenesis. A dose-dependent inhibition of angiogenesis was observed for 1 to 20 μg/CAM. At a dose of 20 μg/CAM (n = 7), there was a sustained inhibition of angiogenesis by two separate batches of aaAT. No hemorrhage was seen in any of the treated groups, and there was no evidence of any toxicity or inflammatory reaction.

To investigate whether aaAT inhibits tumor growth in mice, we injected the SK-NAS cells subcutaneously into immunocompromised severe combined immunodeficiency disease (SCID) mice. After tumors formed, mice were treated with daily subcutaneous injections of the different conformations of antithrombin. Control animals were injected with human stressed antithrombin or vehicle alone. The stressed conformation caused local bleeding but did not substantially affect tumor growth. In contrast, cleaved (bovine or human) and locked (human) antithrombin potently inhibited tumor growth (Fig. 4). Tumors regressed to small subcutaneous nodules without evidence of bleeding or toxicity in any of the mice. Tumors in the control groups were highly vascularized, as shown by immunohistochemical staining with antibodies against von Willebrand factor. In contrast, the small tumors in the treated group contained only microscopic foci of malignancy and were sparsely vascularized. This same pattern of regression of established tumors has been demonstrated previously for human angiostatin (*18*) and endostatin (*19*) but required substantially higher and more frequent dosing.

In a separate experiment, SCID mice implanted with the SK-NAS cells were treated with latent or locked human antithrombin at a dose of 15 mg kg^{-1} day^{-1} (Fig. 4B), and both potently inhibited tumor growth. To rule out the possibility that an undetected contaminant in the native antithrombin preparations was responsible for the antiangiogenic and antitumor effect, we treated mice bearing SK-NAS tumors or Lewis lung carcinomas with stressed, cleaved, or locked recombinant human antithrombin. Cleaved recombinant antithrombin potently inhibited capillary endothelial cell proliferation in vitro (*8*) and tumor growth in vivo (Fig. 4). These data show that aaAT is a potent inhibitor of angiogenesis and tumor growth and demonstrate a previously unknown function for the cleaved conformation of the serpin antithrombin.

In our small-cell lung cancer model, antithrombin is a substrate for an unknown enzyme produced by the tumor cells. Although few studies have been performed, a literature review shows at least one example of a hepatocellular carcinoma that directly expresses antithrombin (*20*). However, other cancers, including small-cell lung cancer, can sequester antithrombin into their stroma (*21*, *22*). Production of aaAT by a malignant tumor may therefore be similar to production of angiostatin from plasminogen. Plasma proteins leaking from tumor vessels form a neostroma (*23*, *24*) for tumor growth but may also mobilize angiogenesis inhibitors. The degradation of antithrombin or plasminogen by such a tumor could induce a deficiency of these proteins that could explain the hypercoagulable state observed with many cancers and the therapeutic benefit of heparin or warfarin therapy. We speculate that the administration of antithrombin or plasminogen could be beneficial not only to correct the deficiency but also to provide increased substrate for conversion to

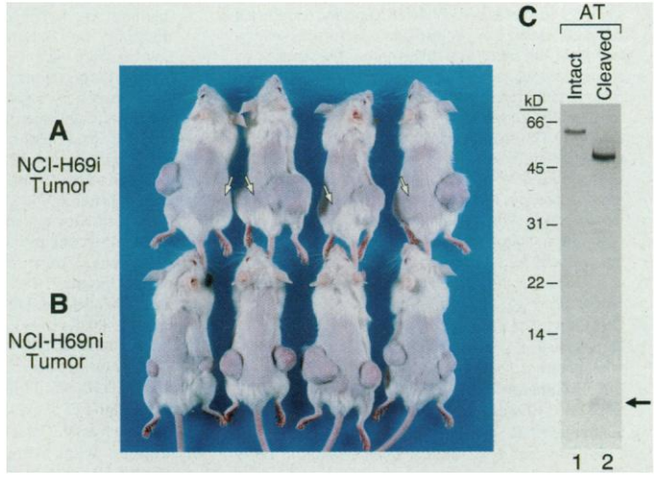

Fig. 1. Identification of an inhibitor of angiogenesis in an in vivo model of tumor growth inhibition by tumor mass. (**A**) By selective in vivo passage, a variant (H69i) of NCI-H69 small-cell lung cancer was developed in which a primary flank tumor completely suppresses the growth of a second implant on the opposite flank (arrows). (**B**) In another variant (H69ni) that does not produce aaAT, no evidence of inhibition of the growth of one tumor by the other is observed. (**C**) SDS-PAGE (reduced, silver stain) of intact (lane 1) and cleaved (lane 2) antithrombin purified from the conditioned media of H69i spheroids. The B chain of the cleaved form is indicated with an arrow.

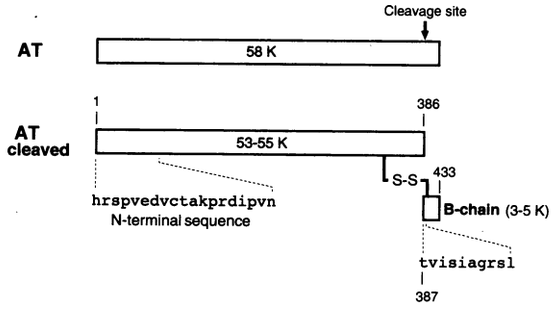

Fig. 2. Sequence data and schematic of bovine antithrombin. NH_2-terminal (N-terminal) sequences were determined by automated Edman degradation on a PE/ABD Procise 494cLC protein sequencer (Foster City, California) with high-sensitivity phenylthiohydantoin amino acid detection by capillary HPLC. Sequence library searches and alignments were performed against combined GenBank, Brookhaven Protein, SWISS-PROT, and PIR databases. K is kilodalton. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; G, Gly; H, His; I, Ile; K, Lys; L, Leu; N, Asn; P, Pro; R, Arg; S, Ser; T, Thr; V, Val; and Y, Tyr.

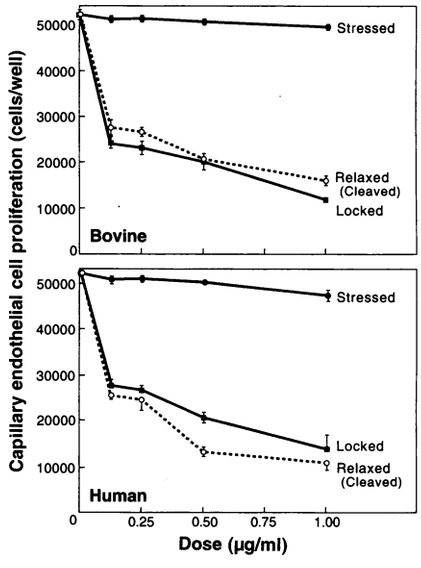

Fig. 3. Inhibition of capillary endothelial cell proliferation by locked or relaxed (cleaved) bovine or human antithrombin.

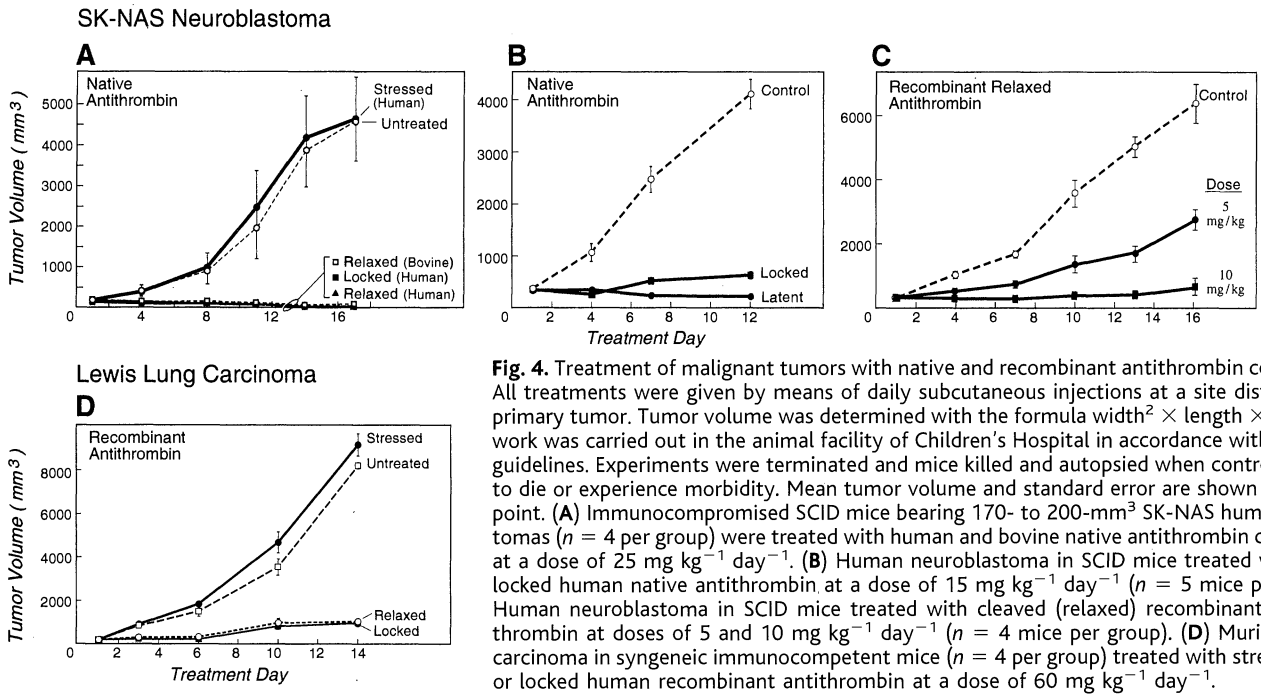

Fig. 4. Treatment of malignant tumors with native and recombinant antithrombin conformations. All treatments were given by means of daily subcutaneous injections at a site distant from the primary tumor. Tumor volume was determined with the formula width^2 × length × 0.52. Animal work was carried out in the animal facility of Children's Hospital in accordance with institutional guidelines. Experiments were terminated and mice killed and autopsied when control mice began to die or experience morbidity. Mean tumor volume and standard error are shown for each time point. (**A**) Immunocompromised SCID mice bearing 170- to 200-mm^3 SK-NAS human neuroblastomas (n = 4 per group) were treated with human and bovine native antithrombin conformations at a dose of 25 mg kg^{-1} day^{-1}. (**B**) Human neuroblastoma in SCID mice treated with latent or locked human native antithrombin at a dose of 15 mg kg^{-1} day^{-1} (n = 5 mice per group). (**C**) Human neuroblastoma in SCID mice treated with cleaved (relaxed) recombinant human antithrombin at doses of 5 and 10 mg kg^{-1} day^{-1} (n = 4 mice per group). (**D**) Murine Lewis lung carcinoma in syngeneic immunocompetent mice (n = 4 per group) treated with stressed, relaxed, or locked human recombinant antithrombin at a dose of 60 mg kg^{-1} day^{-1}.

aaAT or angiostatin. Antiangiogenic antithrombin and other endogenous inhibitors of angiogenesis may thus function to regulate malignant growth.

The generation of aaAT provides additional evidence that clotting factors play a major role in angiogenesis. The presence of inhibitors of angiogenesis within proteins such as plasminogen (*3*), thrombospondin (*25*), platelet factor 4 (*26*, *27*), kininogen (*28*), prothrombin (*29*), and antithrombin may allow for the precise regulation of angiogenesis at sites of microvascular injury. Their mobilization may help balance the proangiogenic response seen after wounding and in malignancy. It remains to be seen whether aaAT will have clinical efficacy. However, aaAT and other angiogenesis inhibitors offer the potential for improved efficacy and diminished toxicity in the treatment of cancer and other angiogenesis-dependent diseases.

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5. H69i and H69ni cell lines were established in vitro and grown as loosely adherent spheroids in 80 ml of Dulbecco's modified Eagle's medium with 2.5% heat-inactivated fetal bovine serum and 1% glutamine penicillin-steptomycin in 900-cm^2 roller bottles. After 96 hours at 37°C and 10% CO_2, media was collected, centrifuged, filtered (0.45 μm), and stored at 4°C. Pooled conditioned medium was diluted 1:3 with 10 mM tris-HCl (pH 7) and applied to a CM Sepharose column coupled to a DEAE Sepharose column. The columns were uncoupled and eluted with a step gradient of NaCl in 10 mM tris-HCl with 50 mM, 0.2 M, 0.6 M, 1 M, and 2 M steps. Fractions with evidence of protein by absorbance at 280 nanometers were pooled, and a portion of each was applied to bovine capillary endothelial cells in vitro. The 0.2 M NaCl elution from the DEAE column inhibited proliferation and was diluted 1:2 and applied to a heparin Sepharose column equilibrated with 0.2 M NaCl and 10 mM tris-HCl. The column was eluted with a continuous gradient of 0.2 to 2 M NaCl in 10 mM tris-HCl. Fractions that inhibited capillary endothelial cell proliferation were pooled and concentrated with a NanoSpin 10K centrifugal concentrator. The sample was applied to a Sephacryl S200 HR column and eluted with phosphate-buffered saline (PBS). Inhibitory fractions were pooled, concentrated, and applied to a SynChropak RP-4 HPLC column equilibrated with H_2O and 0.1% trifluoroacetic acid (TFA). Bound protein was eluted with a gradient of acetonitrile in TFA, and a portion of each fraction was evaporated by vacuum centrifugation, resuspended in PBS, and applied to capillary endothelial cells. The inhibitory activity was purified to apparent homogeneity by subsequent cycles on the C4 column.
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16. Stressed bovine and human antithrombin were obtained from Sigma or Calbiochem, respectively, or purified as described. Recombinant human antithrombin was obtained as a generous gift from a joint venture between Genzyme and Genzyme Transgenics Corporations. Stressed human antithrombin was cleaved by incubation with porcine pancreatic elastase (Calbiochem) for 12 hours at 37°C in PBS (pH 7.8) at a 1:50 molar ratio. Cleaved and latent antithrombin were purified with heparin Sepharose and eluted at 0.4 M NaCl as a distinct peak. Purity was assessed by SDS-PAGE, and samples were concentrated with a Nanospin 30K centrifugal concentrator or lyophilized after dialysis.
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