

Generation of Multiple Angiogenesis Inhibitors by Human Pancreatic Cancer¹

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ABSTRACT

A primary inoculum of human pancreatic cancer cells (BxPC-3) has the ability to inhibit the growth of a secondary tumor in an *in vivo* animal model. Such ability suggests that the primary tumor is producing inhibitors that act at the site of the secondary tumor. Accordingly we attempted to discover which inhibitors are produced by pancreatic cancer cells. We determined that pancreatic cancer cells process angiotatin isoforms from plasminogen. Additionally, we isolated and characterized an uncleaved "latent" antiangiogenic antithrombin (aaAT) molecule processed from systemically available AT by pancreatic cancer cells as well as a cleaved form of aaAT processed from systemically available AT by pancreatic cancer cells. Human AT, cleaved with human neutrophil elastase, inhibits angiogenesis in the chorioallantoic membrane assay. This human aaAT molecule is able to inhibit the growth of pancreatic tumors in immunocompromised mice. Our work represents the first demonstration of multiple angiogenesis inhibitors from a single tumor and suggests that antiangiogenic therapies may provide an avenue for future treatment of pancreatic cancer.

INTRODUCTION

Angiogenesis, the process whereby new blood vessels are induced to "bud" from preexisting vessels, is of profound importance in the transition of tumors from a dormant state to a malignant state (1, 2). Tumors cannot grow beyond a certain size, generally 1–2 mm³, because of a lack of oxygen and other essential nutrients (3). Growth factors, such as bFGF³ and VEGF can induce capillary growth into the tumor, supplying required O₂, nutrients, and allowing the tumor to expand. Thus angiogenesis is a necessary and required step for transition from a small harmless cluster of cells to a large tumor (4). Angiogenesis is also required for the spread of a tumor or metastasis (5). The subsequent growth of such metastases will also require a supply of nutrients and oxygen. The suppression of a secondary tumor by a primary mass has been long studied. Rapid growth of metastases after removal of breast (6) or colon (7) cancer is a well-documented phenomenon. Additionally, tumors of one type can often inhibit the growth of another tumor mass implanted in a distal location contemporaneously (8). Recently it has been postulated that this phenomenon can be explained by a mechanism in which the primary tumor inhibits angiogenesis in remote metastases thereby inhibiting their growth (9).

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³ The abbreviations used are: bFGF, basic fibroblast growth factor; AT, antithrombin; aaAT, antiangiogenic antithrombin; thrombin-AT, thrombin antithrombin complex; VEGF, vascular endothelial growth factor; BCE, bovine capillary endothelial cell; BCS, bovine calf serum; TUG, transverse urea gradient; HNE, human neutrophil elastase; MMP, matrix metalloproteinase; CAM, chorioallantoic membrane.

Such inhibition of angiogenesis is thought to be achieved via the generation of circulating endothelial cell-specific inhibitors by the primary tumor mass (10). Consequently, removal of certain primary tumors can often result in rapid growth of metastases undetected previously, because the angiogenic inhibitor keeping metastases in a dormant state is also removed. This phenomenon has also been observed in mouse models, where a primary tumor on the flank of an immune-compromised mouse can inhibit the growth of a secondary tumor on the opposite flank (11). This *in vivo* model has been used with success to screen tumor lines for the ability to produce or generate angiogenesis inhibitors (12). Recent studies have described such antiangiogenic inhibitors generated by cancer cells. Three examples are angiotatin (13, 14), endostatin (15, 16), and aaAT (12). Here we present data indicating that a cancer can make more than one of these angiogenesis inhibitors. We show that human pancreatic cancer can process plasminogen to multiple isoforms of angiotatin. We also describe the isolation of multiple antiendothelial conformations of AT processed from systemically available AT by the same human pancreatic cancer cell line. This cell line was chosen because of the ability of a primary implant to inhibit the growth of a secondary implant by $\leq 80\%$ in an *in vivo* model using immunocompromised mice. The angiogenesis inhibitors were able to specifically inhibit the proliferation and migration of endothelial cells. Additionally, plasma-derived human AT, treated with HNE, was able to inhibit angiogenesis in the CAM assay and the growth of pancreatic tumors in an *in vivo* mouse model. Our data suggest that tumors, which are known to produce a variety of angiogenic stimulators, may also be capable of producing a spectrum of angiogenesis inhibitors.

MATERIALS AND METHODS

Cell Culture and Conditioned Medium Collection. Human pancreatic adenocarcinoma cell lines BxPC-3, Capan-1, and ASPC-1 (American Type Culture Collection, Rockville, MD) were incubated in RPMI 1640 (Life Technologies, Inc.) supplemented with 10% FCS (Life Technologies, Inc.), 100 $\mu\text{g/ml}$ penicillin G, and 100 $\mu\text{g/ml}$ streptomycin (Pharmacia, Upsal, Sweden). Cells were maintained in T-75 tissue culture flasks (Falcon) and grown in 5% CO₂/95% at 37°C in an humidified incubator. Conditioned medium was produced by adding RPMI 1640/5% FCS (100 ml) to near confluent BxPC-3 cells in 900-cm² roller bottles (Corning) and incubating for 72 h. Conditioned medium was collected, filtered (0.45 μm), and stored at 4°C. Serum-free conditioned medium was generated by the addition of the corresponding serum-free medium to 90–95% confluent cells after washing twice with PBS. After 24 h of incubation, the serum-free conditioned medium was collected, filtered, and used immediately.

BCE Assays. The antiendothelial activity of protein fractions obtained during purification procedures was determined using a proliferation assay. BCE cells were maintained in DMEM with 10% heat-inactivated BCS, antibiotics, and 3 ng/ml recombinant human bFGF (Scios Nova, Sunnyvale, CA). Cells were washed with PBS and dispersed in a 0.05% solution of trypsin. A cell suspension was made with DMEM/10% BCS/1% antibiotics and the concentration adjusted to 32,000 cells/ml. Cells were plated onto gelatinized 24-well culture plates (0.5 ml/well) and were incubated at 37°C in 10% CO₂ for 24 h. The medium was replaced with 0.5 ml of DMEM/5% BCS/1%

antibiotics, and the test sample applied. After 20-min incubation, bFGF was added to each well (1 ng/ml). After 72 h, cells were dispersed in trypsin, resuspended in Isoton II (Fisher Scientific, Pittsburgh, PA), and counted by Coulter counter.

Cell migration was determined in the 48-well modification of the Boyden chamber assay (Neuroprobe Inc., Cabin John, MD). Boyden chambers were assembled by adding 5% FCS to base wells, separated from top wells by an 8- μ m pore Nucleopore PVP-free polycarbonate membrane (Corning Separations, Acton, MA), and precoated with 1.5% gelatin (Difco, Detroit, MI) solution. Preincubated cell suspension ($1 \times$ test sample plus 3×10^5 cells) was pipetted into the upper chamber at 1.5×10^4 cells/well. The chamber was then incubated for 4 h at 37°C in a 10% CO₂ humidified incubator. Membranes were fixed, stained using Gill's Hematoxylin No. 3, and nonmigrated cells removed by scraping. Cell migration was determined microscopically, with four wells/condition.

Purification of Angiostatin from Pancreatic Cancer Cell-conditioned Medium. Plasminogen was incubated (24–48 h at 37°C) with serum-free conditioned medium obtained from cancer cells. Angiostatin was purified by applying this material to lysine Sepharose (Pharmacia) equilibrated in 50 mM Tris (pH 7.4) and eluting lysine binding fragments using 2–3-column volumes of 200 mM ϵ amino caproic acid/50 mM Tris (pH 7.4). Angiostatin isoforms were further purified using a Superdex 75 (Pharmacia) gel filtration column.

Purification of Antiangiogenic Uncleaved AT from Pancreatic Cancer Cell-conditioned Medium. Conditioned medium obtained from BxPC-3 cells were applied to a heparin Sepharose column equilibrated previously with 50 mM of Tris (pH 7.4), and fractions were eluted in incremental steps (0.5 M) with 2.0 M of NaCl. Fractions were assayed for antiendothelial activity using the BCE proliferation assay and additionally purified using a Superdex 200 column equilibrated previously in 50 mM of Tris (pH 7.4).

Generation of Cleaved AT Conformation by BxPC-3 Cells. Human AT was purified using a combination of heparin Sepharose chromatography and anion exchange chromatography. Human AT (5 mg) was added to 500 ml of serum-free conditioned medium conditioned by pancreatic tumor cells. This material was then incubated at 37°C in a roller bottle incubator (Firma) for 72 h. Cleaved human antiangiogenic AT was repurified using a combination of heparin-Sepharose chromatography and anion exchange chromatography.

Affinity Precipitation of Cleaved AT from Tumor-bearing SCID Mice. Blood from SCID mice bearing BxPC-3 tumors (volume ~500 mm³) was collected (by cardiac puncture) into citrated glass vessels, and plasma was obtained by centrifugation. Plasma (100 μ l) was then diluted 10-fold into 50 mM of Tris (pH 7.4) and incubated with 500 μ l of a 50% slurry of heparin Sepharose beads in 50 mM Tris (pH 7.4) buffer for 1 h at room temperature. The beads were then centrifuged, the supernatant discarded, and the beads washed with 50 mM of Tris/500 mM of NaCl (pH 7.4). After an additional centrifugation step, the supernatant was analyzed for the presence of cleaved AT using a combination of reducing SDS-PAGE and Western blotting.

Electrophoresis and Western Blotting. Protein was resolved by SDS-PAGE (4–20% gels; Bio-Rad) and transferred to Immobilon (17). AT was detected using a polyclonal antibody raised against human AT (Affinity Biologicals Inc., Hamilton, Ontario, Canada). Angiostatin was detected using a monoclonal antibody specific for kringle 1–3 of human plasminogen. This antibody was generated within our laboratory and is a conformation-specific reagent. Visualization was by the enzyme chemiluminescence method (Pharmacia). Amino acid sequence was determined from proteins transferred onto Immobilon from SDS-PAGE (18). Proteins were analyzed by TUG gel electrophoresis (19).

CAM Assay. Chick embryos were prepared as reported (20). Sample was dissolved in 10 μ l of Matrigel. Sample dissolved in Matrigel (10 μ l) was microinjected into the outer third of the CAM of day-6 embryos.

HNE Cleavage of Human AT. Human AT was incubated with HNE at a molar ratio of (100:1) for 16 h at 37°C. Cleaved AT was then separated from HNE using anion exchange chromatography.

Animal Studies. All of the animal work was performed in the animal facility at Children's Hospital, Boston, MA, in accordance with federal, local, and institutional guidelines. Male 6–8-week-old immune-compromised mice (SCID; Massachusetts General Hospital, Boston, MA) were acclimated, caged in groups of \leq four in a barrier care facility, and their backs were shaved. All of the mice were fed a diet of animal chow and water *ad libitum*. Animals were anesthetized with methoxyflurane (Pittman-Moore Inc., Mundelein, IL) before

all of the procedures and observed until fully recovered. Animals were euthanized by CO₂ asphyxiation.

Preparation of Tumor Cells for Implantation in Mice. Pancreatic cancer cells and HT 1080 cells grown in cell culture as described above were washed with PBS, dispersed in a 0.05% solution of trypsin, and resuspended in RPMI 1640/5% FCS. After centrifugation (4000 rpm for 10 min at room temperature), the cell pellet was resuspended in RPMI 1640, and the concentration was adjusted to 12.5×10^6 cells/ml. After the site was cleaned with ethanol, tumor cells were injected s.c. with 2.5×10^6 cells in 0.2 ml of RPMI 1640.

Preparation of Lewis Lung Cells for Implantation in Mice. Animals (C57Bl6/J) with Lewis lung carcinoma tumors of 600–1200 mm³ were euthanized, and the skin overlying the tumor was cleaned with Betadine and ethanol. In a laminar flow hood, tumor tissue was excised under aseptic conditions. A suspension of tumor cells in 0.9% normal saline was made by passage of viable tumor tissue through a sieve followed by a series of sequentially smaller hypodermic needles of 22–30-gauge diameter. The final concentration was adjusted to 1×10^7 cells/ml, and the suspension was placed on wet ice. After the site was cleaned with ethanol, mice were injected with 2.5×10^6 cells in 0.2 ml of saline.

Double-Side Tumor Model. A primary tumor of pancreatic cancer cells was generated by injecting 2.5×10^6 cells (0.2 ml) in the left flank. Tumors were measured with a dial-caliper, and volumes were determined using the formula width² \times length \times 0.52. When the tumor volume was \geq 400–500 mm³ (2–2.5% of body weight), which occurred within 14–21 days, the secondary tumor was transplanted into the contra-lateral flank (2.5×10^6 cells in 0.2 ml). Control mice received an identical injection of secondary tumor cells at the same time. Tumors were measured every third day. The extent of suppression of the secondary tumor by the primary tumor was calculated as the difference in tumor volume.

Tumor Cell Implantation and Treatment. Animals were anesthetized via inhalation of isoflurane (Baxter, Deerfield, IL) before all of the surgical procedures and observed until fully recovered. Before tumor cell injection, mice were shaved, and the dorsal skin was cleaned with ethanol. A tumor cell suspension (BxPc 3 or ASPC-1) of 2.5×10^6 cells in 0.2 ml of RPMI 1640 was injected into the s.c. dorsa of mice in the proximal midline. The mice were weighed, and tumors were measured as described above and blinded every 3–5 days in two diameters with a dial-caliper. When the tumor volumes were ~100 mm³ (within 12–14 days for BxPC-3 or 6 days for ASPC-1), mice were randomized into different groups. Experimental groups received HNE-cleaved aaATIII at a dose of 50 mg/kg/day in 0.2 ml of PBS. The control group received comparable injections of saline or uncleaved ATIII. The injections were administered s.c. at a site distant from the tumor. Tumors were measured every third to fifth day, and the ratio of treated to control tumor volume (T:C) was determined for the last time point.

RESULTS

Inhibition of Secondary Tumor Growth by Primary Pancreatic Cancer Tumor. Various pancreatic cancer cell lines were tested for their ability to inhibit the growth of a secondary implant of tumor cells as described in "Materials and Methods." The pancreatic cancer cell line BxPC-3 was able to inhibit the growth of a secondary implant of Lewis lung carcinoma or HT1080 fibrosarcoma by >70% in all of the cases (Table 1). Another pancreatic cell line, Capan-1, was unable to inhibit the growth of a secondary implant of tumor cells using this assay. This suggested that BxPC-3 cells produced a factor(s) that inhibited the growth of the secondary tumor.

Table 1 Inhibition of secondary tumor growth by primary pancreatic cancer tumor

	Lewis lung carcinoma	HT1080 fibrosarcoma
BxPC3		
Control tumor volume (mm ³)	2228.5 \pm 281.1	849 \pm 110
Suppressed tumor volume (mm ³)	364.2 \pm 122.6	230 \pm 54.2
% suppression	84%	73%
Capan-1		
Control tumor volume (mm ³)	1853 \pm 187.6	812 \pm 179.7
Suppressed tumor volume (mm ³)	1675 \pm 138.2	753 \pm 95.2
% suppression	10%	8%

Plasma from BxPC-3-bearing Mice Can Inhibit the Proliferation of Endothelial Cells. Plasma was obtained from mice bearing BxPC-3 tumors. This plasma was tested for antiproliferative activity using an endothelial cell proliferation assay. Plasma from these mice inhibited the proliferation of endothelial cells in a dose-dependent manner, whereas plasma from control mice did not inhibit the proliferation of endothelial cells (Fig. 1).

Inhibition of Endothelial Cell Proliferation by Conditioned Medium from BxPC-3 Cells. Conditioned medium (containing FCS) obtained from pancreatic cancer cells did not inhibit proliferation of endothelial cells in an *in vitro* assay. This is possibly attributable to the relatively high levels of growth factors (detected by ELISA) such as bFGF (6–8 pg/ml) and VEGF (3000–6000 pg/ml) present in the conditioned medium after 24–48 h. Fig. 2 demonstrates that conditioned medium from BxPC-3 cells can inhibit the proliferation of BCE cells in the presence of a neutralizing antibody to VEGF.

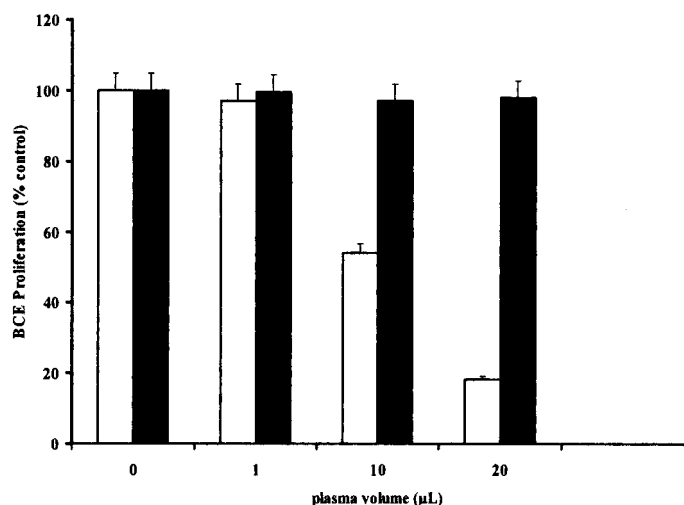


Fig. 1. Plasma from mice bearing BxPC-3 tumors can inhibit the proliferation of endothelial cells. Plasma was obtained by cardiac puncture (see “Materials and Methods”) and assayed as described. Proliferation of BCE cells is inhibited >80% by 20 µl of plasma obtained from mice bearing BxPC-3 tumors (□). Control plasma has no effect on cell proliferation (■); bars, ± SE.

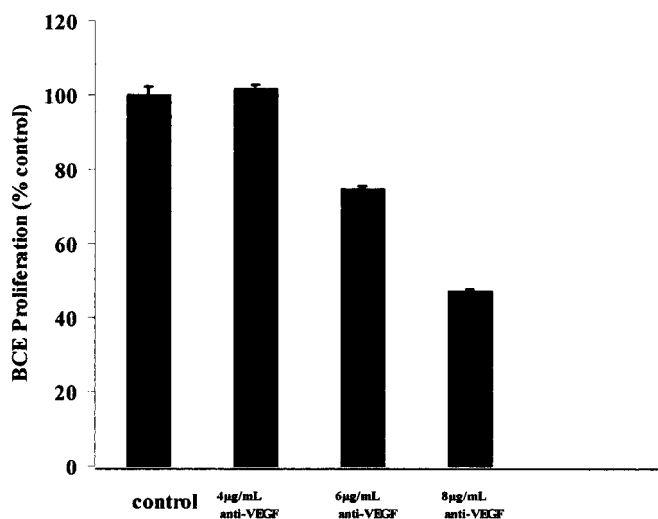


Fig. 2. BxPC-3-conditioned medium inhibits the proliferation of endothelial cells in the presence of anti-VEGF antibody. BxPC-3 conditioned medium does not inhibit the proliferation of endothelial cells (control), presumably because of high VEGF levels (6000–8000 pg/ml). Titrating an anti-VEGF antibody into the proliferation assay can reveal the antiproliferative ability of the conditioned medium. Anti-VEGF antibody alone had no effect on BCE proliferation (data not shown); bars, ± SE.

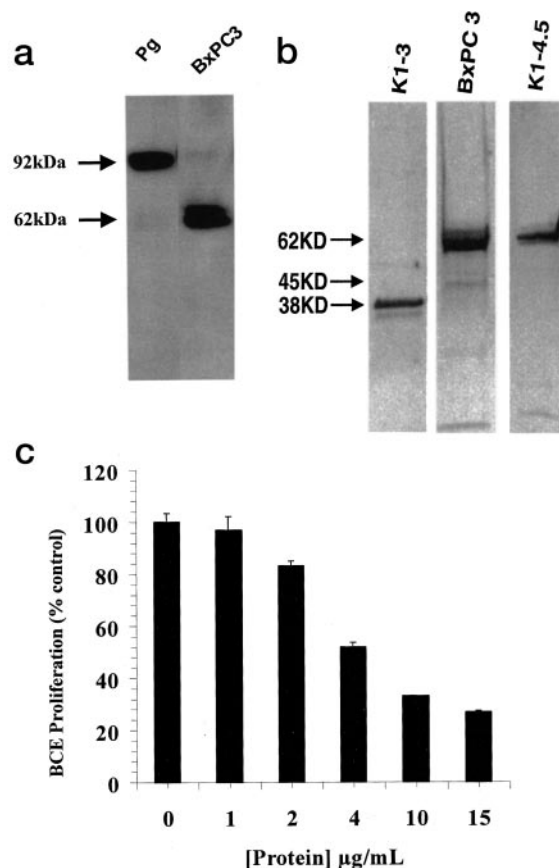


Fig. 3. *a*, Western Blot demonstrating that BxPC-3 cells process plasminogen to angiostatin. Plasminogen (Pg) was incubated with serum-free conditioned medium obtained from BxPC-3 cells (3 mg/100 ml) for 24–48 h at 37°C. Samples were then subjected to nonreducing SDS-PAGE, and the proteins transferred to Immobilon membrane. The blot was probed with a monoclonal antibody specific for kringle 1–3 of human angiostatin. This antibody is conformation specific and does not work under reducing conditions but does cross-react with human plasminogen. Note that plasminogen is processed to a band with apparent M_r 62,000 by BxPC-3 cells. *b*, angiostatin purified from BxPC-3-cell conditioned medium is similar in mass to kringle 1–4.5. Plasminogen was incubated with serum-free conditioned medium obtained from BxPc3 cells. Lysine-binding protein was purified using a combination of lysine-Sepharose affinity chromatography and gel filtration chromatography. Purified protein was analyzed by SDS-PAGE. Note that BxPC-3 cells can generate a major band with M_r 62,000 and a minor band with M_r 45,000. Angiostatin kringle 1–3 was obtained by limited digestion using porcine pancreatic elastase. Angiostatin kringle 1–3 was purified by a combination of lysine-Sepharose chromatography and gel filtration chromatography. Angiostatin kringle 1–4.5 was generated by incubating plasminogen in the presence of uPA and *N*-acetyl cysteine and purifying the resultant angiostatin by affinity chromatography using lysine-Sepharose. *c*, angiostatin purified from BxPC-3 cells inhibits the proliferation of endothelial cells in a dose-dependent manner; bars, ± SE. Incubating human plasminogen with serum-free conditioned medium obtained from BxPC3 cells generated angiostatin. Angiostatin was purified by affinity chromatography using lysine-Sepharose and was assayed using the BCE proliferation assay.

Anti-VEGF alone had no effect on BCE proliferation (data not shown).

Pancreatic Cancer Cells Process Angiostatin from Plasminogen. Pancreatic cancer cells generate angiostatin (21) from available plasminogen. Therefore, we incubated purified human plasminogen with serum-free conditioned medium obtained from pancreatic cancer cells (see “Materials and Methods”). Western blot analysis of these samples (Fig. 3a), using a monoclonal antibody specific for kringles 1–3, indicated that plasminogen was indeed processed to angiostatin by pancreatic cancer cells. Purification of angiostatin by a combination of lysine Sepharose affinity chromatography and gel filtration chromatography resulted in a fraction containing two isoforms of angiostatin (Fig. 3b). These two isoforms appear to correspond to kringle 1–4.5 (14) and angiostatin kringles 1–4 (13). The fraction

containing these two isoforms of angiostatin inhibited proliferation of endothelial cells in a dose-dependent manner (Fig. 3c).

Purification of Uncleaved Antiangiogenic AT from Pancreatic Cancer Cell-conditioned Medium. Conditioned medium (containing FCS) obtained from pancreatic cancer cells was fractionated using heparin Sepharose affinity chromatography as described (“Materials and Methods”). Antiendothelial activity was found in a fraction eluting from the heparin Sepharose column between 1–1.5 M of NaCl. This fraction was further purified using gel filtration chromatography, and antiendothelial activity coeluted with a single band at M_r 58,000–60,000 on SDS-PAGE. Amino-terminal sequence analysis of this band identified it as bovine AT. This material had identical mobility compared with control bovine AT under reducing SDS-PAGE conditions. We noted that this antiendothelial bovine AT had an increased retention time on a gel filtration column when compared with bovine AT that had no antiendothelial activity. This suggested that the antiendothelial bovine AT had a more compact conformation, because no size difference was observed under reducing SDS-PAGE when compared with control bovine AT. Such a compact conformation could be generated by insertion of the reactive site loop of bovine AT into the A- β -sheet. Additionally, serpins in the stressed conformation undergo a characteristic unfolding transition when analyzed by TUG gels (19). Control bovine AT (exhibiting no antiendothelial activity) demonstrated the characteristic unfolding profile (Fig. 4a) that is diagnostic of stressed serpins. Antiendothelial bovine AT did not exhibit the characteristic unfolding transition seen in control bovine AT (Fig. 4b).

This material inhibited the migration of endothelial cells in a dose-response manner (Fig. 4c). Fresh medium (containing FCS) did not contain this antiendothelial form of AT (data not shown). Bovine AT (purified from plasma) eluting from heparin Sepharose between 1 M and 1.5 M of NaCl did not inhibit the migration of endothelial cells (Fig. 4c).

Purification of Cleaved Antiangiogenic AT from Pancreatic Cancer Cell-conditioned Medium. A cleaved form of bovine AT has been purified from the conditioned medium of human small cell lung cancer cells (12). Therefore, after fractionation of pancreatic cancer cell-conditioned medium (containing FCS) on heparin Sepharose (see “Materials and Methods”) we tested these fractions for the presence of cleaved AT by Western blot analysis. A cleaved form of AT was found in the fraction of pancreatic cancer-conditioned medium (containing FCS) eluting from heparin between 0 and 0.5 M of NaCl (Fig. 5a). However, this fraction exhibited no antiendothelial activity, presumably because of the elevated concentrations of growth factors that elute from heparin in this fraction. To determine whether pancreatic cancer cells can cleave AT to an antiendothelial conformation we purified human AT from plasma and incubated this material with serum-free medium conditioned by pancreatic cancer cells. A fraction containing cleaved human AT (Fig. 5b) was then repurified using a combination of heparin-Sepharose affinity chromatography and anion exchange chromatography. Uncleaved human AT migrates with an apparent mass of M_r 58,000, whereas cleaved human AT migrates with an apparent mass of M_r 53,000 on reducing SDS-PAGE. The fraction containing the cleaved form of human AT inhibited the migration (Fig. 5c) of endothelial cells.

Presence of Cleaved AT in Plasma of Tumor-bearing Mice. Plasma obtained from tumor-bearing mice was analyzed for cleaved AT as described in “Materials and Methods.” Plasma was incubated with heparin Sepharose beads and bound protein eluted with 0.5 M of NaCl. Plasma obtained from tumor-bearing mice exhibited a cross-reactive band that migrated with an apparent mass of M_r 53,000 (Fig. 6) as well as the uncleaved molecule migrating at M_r 58,000. The higher migrating band that cross-reacts in both samples may be the AT-thrombin complex. Plasma from control SCID mice exhibit little or no cleaved AT in this fraction.

HNE-cleaved AT Inhibits Angiogenesis in the CAM Assay and Inhibits the Growth of Human Pancreatic Cancer Tumors in Mice. Human AT cleaved with HNE (Fig. 7a) inhibits angiogenesis in the CAM assay (Fig. 7c). Interestingly, uncleaved AT also demonstrates inhibition in this assay (Fig. 7d). This may be attributable to cleavage of AT by proteinases within the CAM during the course of the assay (24–72 h). Ovalbumin is structurally a serpin, but the reactive site loop is maintained in a coiled helical conformation and is unable to insert into the A- β -sheet adopting the relaxed, antiangiogenic conformation (22). Accordingly we used ovalbumin as a negative control in this assay (Fig. 7b).

To test the antitumorigenic function of cleaved AT *in vivo*, mice were implanted with the slow-growing pancreatic cancer BxPC-3 and the fast-growing pancreatic cancer ASPC-1 as described in “Materials and Methods,” and the tumors were allowed to attain a volume of 100 mm³. Mice were randomized into two or three groups. One group received subcutaneous injections of HNE-cleaved human AT at a site distal to the implant, and the control groups received either subcutaneous injections of saline or uncleaved human AT. At a dose of 50 mg/kg/day, HNE-cleaved human AT (Fig. 7e) maintained dormancy of the BxPC-3 tumor over a period of 21 days. Cleaved AT inhibited the growth of ASPC-1 (Fig. 7e) compared with the saline control and the uncleaved AT control over a period of 11 days. At the end of 11 days, animals bearing ASPC-1 tumors were euthanized because of the large size of untreated tumors and tumor necrosis.

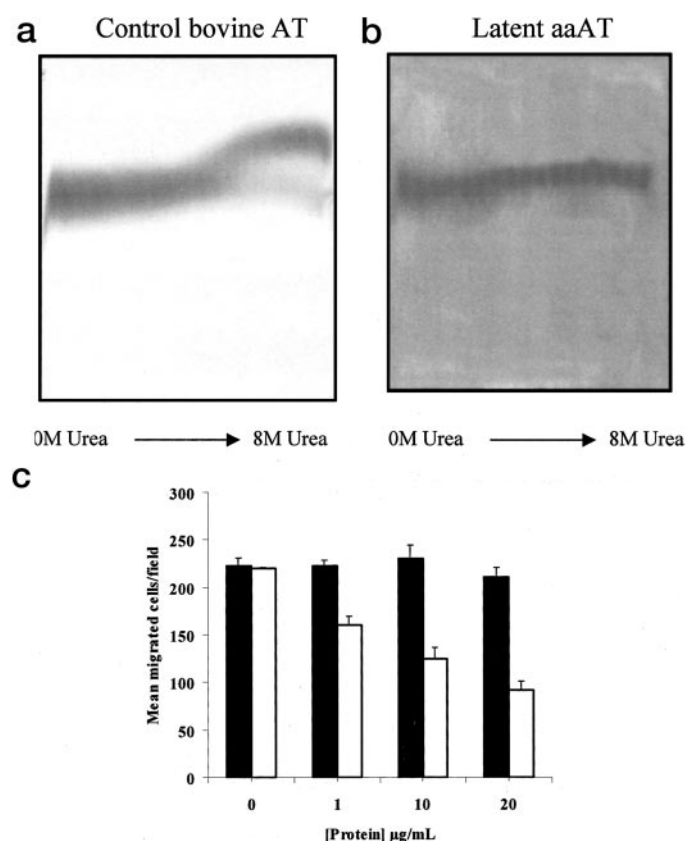


Fig. 4. a and b, aaAT generated by BxPC-3 cells does not undergo an unfolding transition on TUG gel electrophoresis. Control bovine AT (a) and aaAT generated by BxPC-3 cells (b) were analyzed by TUG gel electrophoresis. Control bovine AT undergoes a characteristic unfolding transition at ~ 6 M Urea (a), whereas aaAT (generated by BxPC-3 cells) does not (b). Proteins are visualized with Coomassie Brilliant Blue. c, inhibition of endothelial cell migration by latent aaAT generated by BxPC-3 pancreatic cancer cells. Latent bovine aaAT (\square) was purified from conditioned medium of BxPC3 cells as described (see text). Antimigratory activity was assessed as described in “Materials and Methods.” Control protein (\blacksquare) was bovine AT; bars, \pm SE.

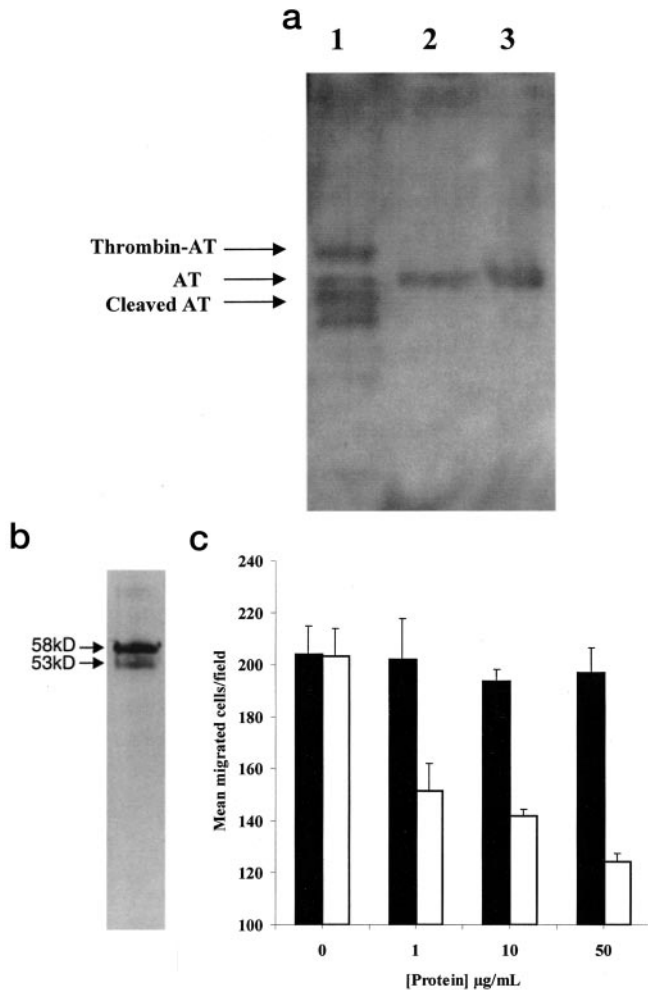


Fig. 5. *a*, BxPC-3 human pancreatic cancer cells generate a cleaved form of AT. Conditioned medium (5% FCS) obtained from BxPC3 cells was fractionated on heparin-Sepharose, and fractions were eluted using 0.5 M of NaCl incremental steps. Fractions were analyzed by Western blot probed with polyclonal antibody raised against AT. Material eluting between 0 and 0.5 M NaCl (Lane 1) contained whole AT, a higher molecular weight species that is likely to be a thrombin-AT complex and reactive site loop-cleaved AT. Note that material eluting at higher heparin affinities 0.5–1.0 M NaCl (Lane 2) and 1–1.5 M NaCl (Lane 3) does not contain either thrombin-AT complex or reactive site loop-cleaved AT. *b*, 3 BxPC-3 human pancreatic cancer cells generate a cleaved form of human AT. Human AT was added to serum-free medium and incubated with BxPC3 cells for 72 h at 37°C. A fraction containing cleaved human AT was then purified using a combination of heparin Sepharose affinity chromatography and anion exchange chromatography. The fraction containing cleaved AT was analyzed by Western blot and probed with a polyclonal antibody raised against human AT to confirm the identity of the cleaved fragment. Uncleaved AT migrates with an apparent mass of M_r 58,000, whereas cleaved AT migrates with an apparent mass of M_r 53,000 on reducing SDS-PAGE. *c*, human cleaved aaAT generated by BxPC-3 cells inhibits the migration of endothelial cells. Human cleaved aaAT (\square), generated by BxPC3 cells and subsequently purified by a combination of heparin affinity and anion exchange chromatography inhibited the migration of endothelial cells in a dose-response manner. Control protein (\blacksquare) was human AT; bars, \pm SE.

DISCUSSION

We demonstrate here that a human pancreatic cancer can generate angiotatin and two forms of antiangiogenic AT from systemically available plasminogen and AT. The predominant angiotatin protein generated by the cancer is similar in mass to kringle 1–4.5. A minor form of angiotatin with a smaller mass also appears to be generated from plasminogen by BxPC-3 cells. It is known that other cancers generate these forms of angiotatin (23). We have demonstrated that this form of angiotatin can inhibit the proliferation of endothelial cells *in vitro*. We have also demonstrated that BxPC-3 cells can convert systemically available AT into two forms of aaAT. One form

is an uncleaved latent conformation, which can inhibit the migration of endothelial cells in a dose-dependent manner. The other is a cleaved form that can also inhibit the migration of endothelial cells in a dose-dependent manner. We also demonstrate that HNE-cleaved AT

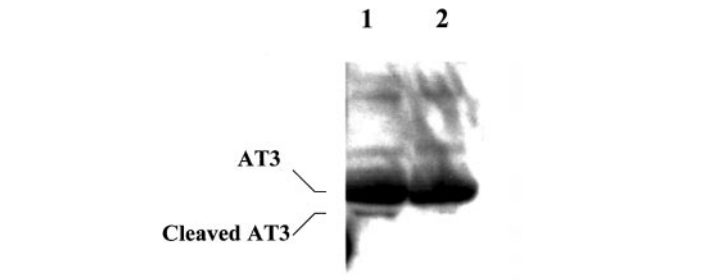


Fig. 6. Plasma obtained from mice bearing BxPC3 tumors contains a cleaved form of AT. Plasma was obtained from mice bearing BxPC3 tumors. AT was affinity precipitated using heparin Sepharose beads. AT was eluted from the beads using 0.5 M of NaCl. These samples were analyzed by reducing SDS-PAGE and Western blotting. Blots were probed with a polyclonal antibody that showed cross-reactivity with murine AT. Note that in plasma from mice with tumors (Lane 1), there appears to be a cross-reactive band corresponding to cleaved AT, whereas no such band appears in control plasma samples (Lane 2).

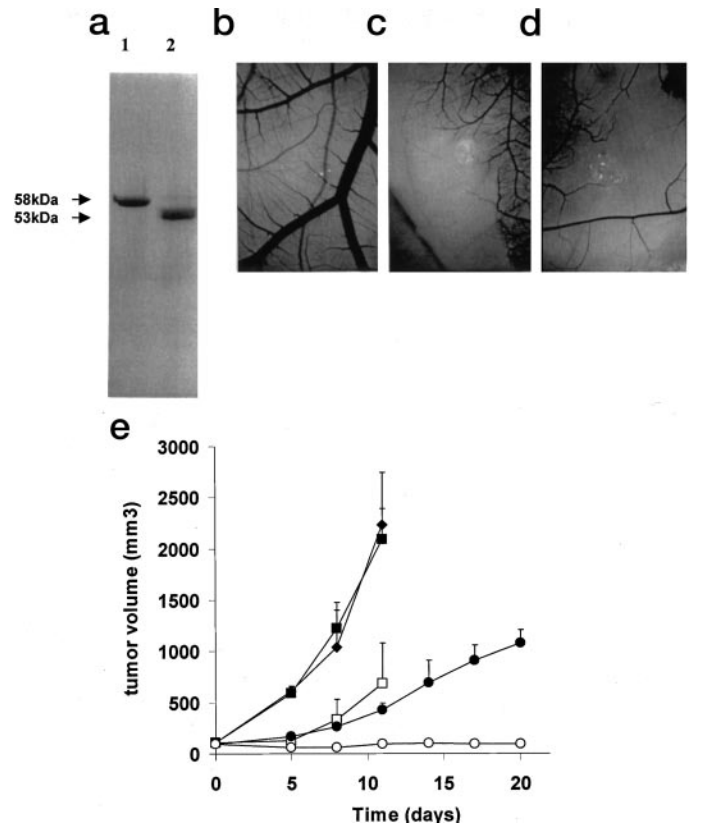


Fig. 7. *a*, human AT cleaved with HNE. Human AT (Lane 1), purified from plasma by a combination of heparin affinity and anion exchange chromatography was cleaved with HNE (Lane 2) as described (see “Materials and Methods”). Samples were analyzed by SDS-PAGE (4–20%) in the presence of β -mercaptoethanol. *b–d*, AT inhibits angiogenesis in the CAM assay. CAM assays were prepared as described (see “Materials and Methods”). Ovalbumin (a serpin that cannot adopt the “relaxed” conformation) does not inhibit angiogenesis in this assay (*b*), whereas cleaved AT (*c*) inhibits angiogenesis. Uncleaved AT inhibits angiogenesis to a lesser degree (*d*), possibly as the result of a cleavage event during the assay. *e*, HNE cleaved aaAT inhibits the growth of human pancreatic tumors *in vivo*. Mice with fast growing (ASPC-1) and slow growing (BxPC-3) human pancreatic tumors were prepared as described (see “Materials and Methods”). Animals with ASPC-1 tumors received s.c injections of saline (\blacksquare), 50 mg/kg/day of uncleaved AT (\blacklozenge), or 50 mg/kg/day of HNE-cleaved AT (\square). Animals with BxPC-3 tumors received either saline (\bullet) or 50 mg/kg/day of HNE-cleaved AT (\circ); bars, \pm SE.

can inhibit angiogenesis in the CAM assay as well as inhibit the growth of human pancreatic tumors in mouse models. Furthermore, although we are unable to show here that BxPC-3 tumor-bearing mice have circulating angiostatin, these mice appear to have detectable circulating levels of aaAT. The inability to detect angiostatin may reflect insufficient sensitivity in the assay used (Western blot).

AT is a member of the structurally related family of proteins termed serpins, which regulate the activity of serine proteinases (24). They function by presenting a reactive site loop to a target proteinase (25). On binding, the serpin undergoes a conformational change whereby the reactive site loop is inserted into the A- β -sheet of the molecule, trapping the proteinase (26). If a serpin cannot form a tight complex with the proteinase, then the serpin is cleaved somewhere within the reactive site loop. Such a cleavage event also allows the reactive site loop to insert into the β -sheet. We do not know the mechanism by which these forms are generated *in vitro* by the pancreatic cells, although we can speculate that a proteinase secreted by the cancer cells is generating the cleaved form. It is known that HNE will cleave at more than one bond within the reactive site loop of human AT and also cleave a peptide from the NH₂-terminal portion of the protein,⁴ accounting for the heterogeneity seen in Fig. 7a (Lane 2). It is not known where the putative proteinase from BxPC-3 cells cleaves within the antiangiogenic bovine AT. We speculate that the latent aaAT generated by BxPC-3 cells may be the result of complex dissociation in the absence of a cleavage event. Complex dissociation to yield uncleaved serpins, although rare, has been reported in the presence of α_2 -macroglobulin (27, 28). We do know that the antiangiogenic forms of AT share a common conformation, the "relaxed" conformation. Thus, the antiangiogenic properties of AT appear to reside in a conformational change rather than a specific cleavage.

Recent experiments have also demonstrated that cleaved and latent AT can inhibit angiogenesis in the CAM assay (29). Our data suggest that uncleaved AT also inhibits angiogenesis in the CAM, although uncleaved AT has no effect on the growth of human pancreatic tumors (Fig. 7e). We speculate that a proteinase present in the chick CAM or developing chick embryo vasculature may be cleaving the AT to an aaAT conformation during the course of this assay. There are also reports demonstrating that latent AT is a more efficient inhibitor of tumor growth than cleaved AT (29). However, in those studies (29), a human protein was injected into immune-competent mice at a 50-fold lower dose than we have used here in SCID mice. It should also be noted that treatment was begun 4 days after tumor cell injection (29). Examination of the data (29) shows that tumor volume was much less than 100 mm³. In contrast, in our experiments tumors were allowed to attain a volume of 100 mm³ before therapy was initiated. Such differences in procedure may in part explain differences in outcome.

Cancer cell lines are known to produce a variety of proangiogenic growth factors such as bFGF and VEGF (30). It is now increasingly likely that cancer cell lines also produce multiple antiangiogenic factors. The presence of multiple inhibitors and multiple isoforms of specific inhibitors in a given cancer is not surprising when one considers that angiogenic inhibitors can be derived from existing systemic proteins by proteolytic processing. For example, among the several proteinases suggested to be responsible for angiostatin generation are macrophage metalloelastase (31), MMP-2, (32), MMP-3, (33), MMP-7, MMP-9 (34), and plasmin itself in the presence of a free sulfhydryl donor such as cysteine (35, 36). Cancer cells are known to produce a variety of MMPs (37) as well as serine proteinases (38).

In common with other angiogenesis inhibitors such as angiostatin

and the kringle domains of prothrombin (39), aaAT is generated from a parent molecule by proteolysis. These parent molecules (plasminogen, AT, and prothrombin) are found within the hemostatic system, suggesting a link between hemostasis and angiogenesis (40). The presence of malignant disease is accompanied frequently by disorders of hemostasis (41). Such disorders are associated with hypercoagulability and hemorrhage. Hemorrhage may be associated with a decrease of a certain coagulation factor, alterations of vascular integrity and platelet numbers, or function in various combinations (42). Thrombin activity associated with cancer cells induces platelet aggregation (43). By depleting functional AT, a tumor may shift the hemostatic system to a hypercoagulable state. This suggests that the many hemostatic defects seen in patients with malignancy may stem, in part, from the depletion of hemostatic proteins by the tumor as it generates angiogenesis inhibitors by a variety of proteolytic mechanisms. Additionally, there is evidence that serpins are cleaved in the course of malignancy. A M_r 41,000 fragment detected in the urine of patients suffering from acute leukemia was found to be a reactive site loop-cleaved form of α_1 proteinase inhibitor (44).

Although we show that mice bearing BxPC-3 tumors have detectable levels of circulating aaAT, it is unclear that this is the sole agent responsible for the inhibition of a secondary tumor. In fact, given the relatively high levels of antiangiogenic human AT necessary for tumor dormancy (~1 mg/mouse/day), it is unlikely that this agent is solely responsible for inhibition of tumor growth in our double-sided tumor model. However, it is apparent that BxPC-3 cells are capable of generating low levels of aaAT *in vivo*, which may be enough to inhibit the vascularization of small secondary tumors.

The death rate from pancreatic cancer is 95% within the first year. The overall survival rate of 3% over 5 years has not been improved over the last 3 decades. Radiation and chemotherapy have only marginal benefit in the treatment of pancreatic cancer. However, our studies demonstrate that a specific inhibitor of angiogenesis, generated by some human pancreatic cancers, can in fact be used as a therapy for pancreatic cancer. These data also show that pancreatic cancer is angiogenesis-dependent, because it can be treated with antiangiogenic therapy. Thus, antiangiogenic therapy may provide an avenue for future treatment of pancreatic cancer.

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⁴ S. Van Patten, personal communication.

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