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Advances in Brief

Human Prostate Carcinoma Cells Express Enzymatic Activity That Converts Human Plasminogen to the Angiogenesis Inhibitor, Angiostatin[1]

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Abstract

Angiostatin is an inhibitor of angiogenesis and metastatic growth that is found in tumor-bearing animals and can be generated *in vitro* by the proteolytic cleavage of plasminogen. The mechanism by which angiostatin is produced *in vivo* has not been defined. We now demonstrate that human prostate carcinoma cell lines (PC-3, DU-145, and LN-CaP) express enzymatic activity that can generate bioactive angiostatin from purified human plasminogen or plasmin. Affinity purified PC-3-derived angiostatin inhibited human endothelial cell proliferation, basic fibroblast growth factor-induced migration, endothelial cell tube formation, and basic fibroblast growth factor-induced corneal angiogenesis. Studies with proteinase inhibitors demonstrated that a serine proteinase is necessary for angiostatin generation. These data indicate that bioactive angiostatin can be generated directly by human prostate cancer cells and that serine proteinase activity is necessary for angiostatin generation.

Introduction

Angiostatin, a proteolytic fragment of plasminogen including kringles 1–4, is a potent inhibitor of angiogenesis and the growth of tumor cell metastases (1). Angiostatin can be generated *in vitro* by limited elastase proteolysis of plasminogen (2, 3) and is found *in vivo* in tumor-bearing mice (1, 3). The enzymatic mechanism by which angiostatin is generated *in vivo* remains unknown. We have shown that lung and liver metastases of PC-3 human prostate carcinoma cells in athymic mice remain at the microscopic stage, whereas the primary tumor increases 4-fold in size (4). These data suggest that PC-3 cells express a factor that suppresses the growth of metastatic tumor cells. The recent demonstration that bFGF[3]-induced corneal angiogenesis is inhibited in mice bearing s.c. PC-3 tumors (5) suggests that the antimetastatic factor is an angiogenesis inhibitor. We now report that PC-3 cells secrete enzymatic activity able to cleave plasminogen to bioactive angiostatin.

Materials and Methods

Cell Culture. The human prostate carcinoma cell lines PC-3, DU-145, and LN-CaP were grown in RPMI 1640 supplemented with 10% fetal bovine serum, 100 units/ml penicillin G, and 100 mg/ml streptomycin (Life Technologies, Inc., Gaithersburg, MD). HUVECs were grown in RPMI supplemented with 20% bovine calf serum (A-2151-L; Hyclone Laboratories, Inc., Logan, UT), 100 units/ml penicillin G, 100 mg/ml streptomycin, 2 mM L-glutamine (Life Technologies, Inc.), 2500 units sodium heparin (Fisher Scientific, Itasca, IL), and 50 mg/ml endothelial cell growth supplement (Collaborative Biomedical Research, Bedford, MA). Cells were maintained at 37°C in a humidified incubator in an atmosphere of 5% CO_2. To generate SFCM, confluent cell monolayers were washed twice with PBS, then serum-free RPMI was added. The next day the SFCM was collected and centrifuged at 3000 rpm for 15 min to remove insoluble cellular debris.

Angiostatin Generation. Two μg of human plasminogen, obtained by lysine-Sepharose affinity chromatography of human plasma (6), or human plasmin (527624; Calbiochem-Novabiochem Corp., La Jolla, CA) were added to 100-μl aliquots of the SFCM, and the mixture was incubated at 37°C overnight. Aliquots were analyzed for angiostatin generation by Western blot (see below). Plasminogen cleavage by SFCM was also assessed in the presence of proteinase inhibitors (Boehringer Mannheim, Indianapolis, IN).

Western Blot. Samples were electrophoresed under nonreducing conditions on 12% polyacrylamide gels (NOVEX, San Diego, CA) in Tris-glycine running buffer (7) and electrotransferred to a 0.45 μM polyvinylene difluoride membrane (Immobilon, Millipore, Bedford, MA). The membrane was then blocked for 30 min in blocking buffer (1% BSA in Tris-buffered saline) and probed with a 1:1000 dilution of a monoclonal antibody to the kringle 1–3 (K1-3) fragment of human plasminogen (VAP 230L, Enzyme Research Laboratories, Inc., South Bend, IN). After being washed, the membrane was incubated for 30 min with an alkaline phosphatase conjugated goat antimouse IgG secondary antibody (Kirkegaard & Perry Laboratories, Gaithersburg, MD) and developed using 5-bromo-4-chloro-3-indoyl phosphate/nitroblue tetrazolium (Kirkegaard & Perry Laboratories).

Zymographic Analysis. Zymograms to detect matrix metalloproteinase activity were performed as described previously (8).

Chromogenic Peptide Substrates. To determine whether a prostate carcinoma cell-derived elastase was present, 50 μl of SFCM were incubated with 0.3 mM of chromogenic peptide substrates specific for elastase (substrate I, MeOSuc-Ala-Ala-Pro-Val-pNA; substrate II, Boc-Ala-Ala-Pro-Ala-*p*NA; substrate III, *p*Glu-Pro-Val-*p*NA; substrate IV, Suc-Ala-Ala-Pro-Abu-*p*NA; Calbiochem-Novabiochem Corp.) at 37°C for 2–18 h. Substrate cleavage was determined by monitoring the absorbance at 405 nm (Molecular Devices, Menlo Park, CA).

Lysine-Sepharose Purification of Angiostatin. To generate purified PC-3-derived angiostatin for bioactivity analyses, human plasminogen was incubated with the PC-3 SFCM at 20 μg/ml overnight at 37°C. The reaction product was applied to a lysine-Sepharose column, preequilabrated with TBS (50 mM Tris, pH 7.5, and 150 mM NaCl). Following washes with TBS to remove non-specifically bound protein, angiostatin was eluted in 0.2 M ϵ aminocaproic acid in TBS. The eluted fraction was dialyzed (molecular weight cutoff, 12,000–14,000) to PBS. To remove residual plasmin, the angiostatin was applied to a soybean trypsin inhibitor agarose (Sigma Chemical Co., St. Louis, MO) column, and the flow-through was collected, filter-sterilized, and stored at −80°C until used. Angiostatin was quantitated by measuring the absorbance at 280 nm, using an extinction coefficient ($A^{1\%}/_{1\ cm}$) of 8.0 (2). The purified angiostatin was also examined by Coomassie Brilliant Blue staining of polyacrylamide gels and immunodetection by Western blot. Elastase-generated

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[3] The abbreviations used are: bFGF, basic fibroblast growth factor; HUVEC, human umbilical vein endothelial cell; SFCM, serum-free conditioned medium.

angiostatin, purified from human plasma, was a generous gift from M. S. O'Reilly (Children's Hospital, Harvard University, Boston, MA).

Microsequence Analysis of PC-3-derived Angiostatin. To determine the NH_2-terminus of the angiostatin bands, 10 μg of the affinity purified PC-3-derived angiostatin was electrophoresed on a 12% SDS-polyacrylamide gel, electroblotted to a polyvinylene difluoride membrane, and stained with Coomassie Blue. The bands were excised, placed on Porton sample support discs, and sequenced using a pulse liquid-phase sequencer with phenylthiohydantoin analysis.

Endothelial Cell Proliferation Assay. Cell proliferation was determined using the CellTiter 96 AQ nonradioactive cell proliferation assay (Promega Corp., Madison, WI). The human endothelial cells were plated in a 96-well tissue culture plates (Becton Dickinson, Lincoln Park, NJ) at a concentration of 5.0×10^3 cells/well. The following day, 1, 5, 8, or 10 μg/ml of angiostatin was added to triplicate wells. Wells without angiostatin served as control. The cells were incubated for 72 h, and an absorbance read at 490 nm, reflecting the number of proliferating cells, was measured using an automated microplate reader (Molecular Devices). The results are reported as a percentage of untreated control cells.

Endothelial Cell Migration Assay. To determine the ability of PC-3-derived angiostatin to block migration of endothelial cells toward the angiogenic factor bFGF, migration assays were performed in a modified Boyden chamber using bovine capillary endothelial cells (a kind gift from Dr. J. Folkman, Harvard Medical School, Boston, MA) as described previously (9). Cells were grown in DMEM with 10% donor calf serum and 100 mg/ml endothelial cell mitogen and used at passage 15. To assess migration, the cells were starved overnight in DMEM supplemented with 0.1% BSA, harvested, suspended in DMEM/BSA, plated at 10^6 cells/ml on the lower surface of a gelatinized membrane (Nucleopore Corp., Plesanton, CA) in an inverted Boyden chamber, and incubated for 1.5–2 h to allow cell attachment. The chambers were reinverted, test material was added to the top well, and the chamber was incubated for an additional 3–4 h. Membranes were then fixed and stained, and the number of cells that migrated to the top of the filter in 10 high-powered fields was determined. DMEM with 0.1% BSA was used as a negative control, and bFGF at 10 ng/ml was used as a positive control.

Endothelial Cell Tube Formation. HUVECs were plated on gels of Matrigel (kindly provided by Hynda Kleinman, National Institute of Dental Research) in 24-well tissue culture plates as described previously (10). PC-3-derived angiostatin in nonconditioned RPMI was added to the wells, followed by cells at a final concentration of 4.0×10^4 cells in 1 ml of 50% HUVEC culture medium, 50% RPMI. Each angiostatin or control condition was assayed in triplicate. The cultures were incubated for 16–18 h at 37°C in a 5% CO_2 humidified atmosphere, then fixed with Diff-Quick Solution II (Baxter, McGaw Park, IL). A representative area of the tube network was photographed using a Polaroid MicroCam camera at a final magnification of ×35. The photographs were then quantitated by a blinded observer who measured the length of each tube, correcting for portions of tubes that were incomplete. The total length of the tubes was determined for each photograph and the mean tube length was determined. The results were expressed as the mean ± SE.

Corneal Angiogenesis Assay. The corneal assay was performed as described previously (11). Briefly, 5-μl hydron pellets (Hydron Laboratories, New Brunswick, NJ) containing 10 μg/ml bFGF or bFGF plus 1 or 10 μg/ml angiostatin were implanted into the cornea of anesthetized rats. After 7 days, the animals were sacrificed, corneal vessels were stained with colloidal carbon, and corneas were examined for angiogenic activity.

Results and Discussion

Angiostatin Generation by Prostate Cancer Cells. Incubation of human plasminogen with PC-3 cell-derived SFCM resulted in the generation of multiple immunoreactive bands at approximately 50 kD (Fig. 1*A*), similar to those observed by O'Reilly *et al.* (1). Examination of SFCM from two additional human prostate carcinoma cell lines, DU-145 and LN-CaP, also revealed the generation of the multiple bands, similar to the PC-3 SFCM (data not shown). The initial indication that the product was angiostatin was based on the immunoreactivity with the monoclonal antibody specific for kringles 1–3 (K1-3) of plasminogen and the size of the cleavage product that approximated the predicted mass of kringles 1–4 of human plasminogen. Subsequent confirmation that the prostate carcinoma-derived plasminogen cleavage product was bioactive angiostatin is described below.

Angiostatin generation by PC-3 SFCM was time-dependent; there was a significant decrease in the plasminogen substrate and a corresponding increase in angiostatin beginning at 3 h, with complete conversion to angiostatin by 24 h (Fig. 1*B*). Dilution of the PC-3 SFCM resulted in a proportional decrease in angiostatin generation (Fig. 1*C*). To determine whether plasmin, the activated form of the zymogen plasminogen, could also be converted to angiostatin, we evaluated plasmin as a potential substrate for PC-3-derived angiostatin-generating activity. Incubation of plasmin with SFCM yielded a product indistinguishable from the plasminogen-derived angiostatin (Fig. 1*A*). In kinetic studies, plasmin was converted to angiostatin at a rate comparable to that of the plasminogen; 50% conversion by 8 h, with complete conversion by 24 h (data not shown). These data suggest that *in vitro*, both plasminogen and plasmin are substrates for angiostatin generation.

Enzymatic Class of Plasminogen-Angiostatin Converting Activity. To determine the proteolytic class of the angiostatin-generating activity, PC-3 SFCM was incubated with plasminogen in the presence of various proteinase inhibitors. Only serine proteinase inhibitors

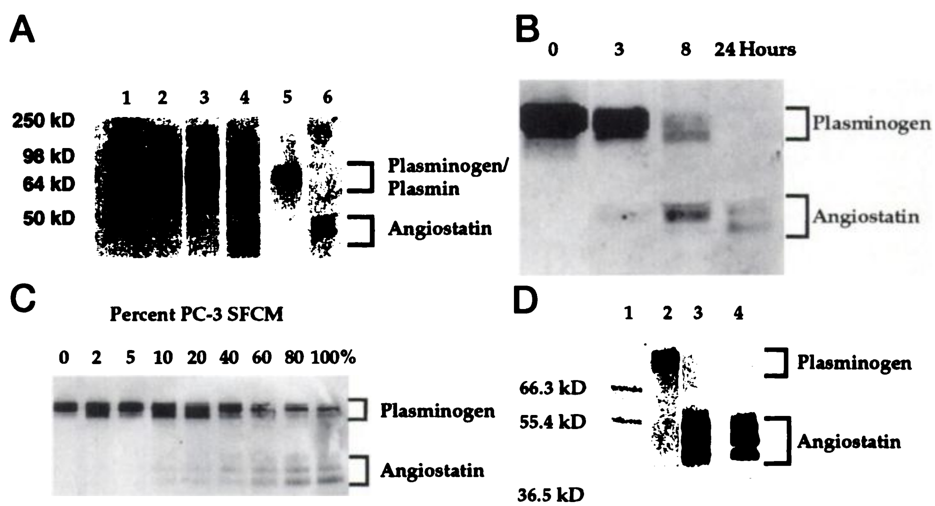

Fig. 1. Conversion of plasminogen and plasmin to angiostatin by PC-3 SFCM. *A*, *Lane 1*, molecular weight standard; *Lane 2*, human plasminogen; *Lane 3*, human plasminogen incubated overnight at 37°C in nonconditioned RPMI; *Lane 4*, human plasminogen incubated overnight at 37°C in SFCM from PC-3 cells; *Lane 5*, human plasmin incubated in nonconditioned RPMI; *Lane 6*, human plasmin incubated in SFCM from PC-3. *B*, the generation of angiostatin from plasminogen was time dependent. PC-3 SFCM was incubated with plasminogen, and at the time points indicated, aliquots were removed and snap frozen prior to Western blot analysis. Trace generation of angiostatin was first observed at 3 h, and complete conversion was noted at 24 h. *C*, generation of angiostatin by PC-3 SFCM was concentration dependent. SFCM was diluted with fresh RPMI and incubated with plasminogen for 24 h. *D*, affinity purification of PC-3 SFCM-derived angiostatin. *Lane 1*, molecular weight standard; *Lane 2*, human plasminogen incubated overnight at 37°C in nonconditioned RPMI; *Lane 3*, PC-3-derived angiostatin, affinity purified on lysine-Sepharose and stained with Coomassie Blue; *Lane 4*, PC-3-derived affinity purified angiostatin detected on Western blot using the K1-3 monoclonal antibody.

blocked angiostatin generation (see Table 1). In contrast, none of the other classes of proteinase inhibitors were effective. Angiostatin can be generated *in vitro* by limited proteolysis of plasminogen by elastase (2, 3, 12). In the present study, angiostatin generation was not inhibited by elastatinal, a specific inhibitor of elastase (see Table 1). Additionally, no elastase activity was detected in PC-3 SFCM based on coincubation of SFCM with four elastase-sensitive chromogenic substrates for 24 h (not shown). These data indicate that the human plasminogen-angiostatin converting activity is unlikely to depend on the action of an elastase. Furthermore, gelatin zymograms revealed no evidence of active or latent metalloproteinases in the PC-3 SFCM (not shown).

Purification of PC-3-derived Angiostatin. PC-3-derived angiostatin was affinity purified on lysine-Sepharose (3), and the resulting product was examined by Western blot and Coomassie Blue staining (Fig. 1*D*). The amino-terminal sequence of all three bands was KVYLSECKTG, which corresponds to residues 78–87 of the plasminogen molecule, confirming that the product was an internal fragment of plasminogen.

PC-3-derived Angiostatin Inhibits Angiogenesis. Because angiogenesis represents a cascade of cellular processes that includes endothelial cell proliferation, migration, and tube formation (13), we used multiple *in vitro* and *in vivo* assays related to angiogenesis to confirm that the PC-3-derived product was bioactive angiostatin. Affinity purified PC-3-derived angiostatin inhibited human endothelial cell proliferation in a concentration-dependent manner; significant inhibition was observed at 10 μg/ml ($P < 0.05$), in comparison to the untreated control cell proliferation (Fig. 2*A*). PC-3-derived angiostatin also inhibited the bFGF-induced migration of bovine capillary endothelial cells (Fig. 2*B*) with an ED_{50} of 0.35 μg/ml. The dose/response curve of PC-3-derived angiostatin was indistinguishable from that of elastase-generated angiostatin purified by O'Reilly (3). Inhibition of migration occurred at a 10-fold lower concentration than required to inhibit proliferation, a finding that has been reported for other inhibitors of angiogenesis (14). This may be due to the fact that the proliferation assay, in contrast to the migration assay, was conducted in RPMI supplemented with 20% calf serum and endothelial cell growth supplement, and therefore contained multiple stimulatory factors. Endothelial cell tube formation on Matrigel was significantly inhibited at 15 μg/ml (Fig. 3, *A* and *B*); the mean length of tubes in the untreated control was 674.5 ± 54 mm, in comparison to the length of tubes exposed to PC-3-derived angiostatin, 287.7 ± 47 mm ($P < 0.005$).

To determine the effect of PC-3-derived angiostatin on corneal angiogenesis *in vivo*, its ability to block bFGF-induced angiogenesis was tested. The bFGF pellet induced angiogenesis in 100% of implanted corneas (Fig. 3*C*). In contrast, angiostatin at 10 μg/ml completely inhibited the bFGF-induced angiogenic response in three of three animals (Fig. 3*D*). At a lower dosage (1.0 μg/ml), angiostatin completely blocked angiogenesis in two of three animals, with partial inhibition in the third animal. Taken together, these data indicate that the angiostatin generated by the PC-3 SFCM is a potent inhibitor of both *in vitro* and *in vivo* angiogenesis.

These data demonstrate that human prostate carcinoma cells express plasminogen-angiostatin converting enzyme activity that is detectable and stable in the SFCM. The enzymatic activity necessary for angiostatin generation was shown to require a serine proteinase but not an elastase isoform and has been preliminarily designated plas-

Table 1 *Proteinase inhibitors*

The proteinase inhibitors were added to the SFCM/plasminogen mix prior to the overnight incubation. Samples were analyzed by Western blot for evidence of inhibition of angiostatin generation.

Proteinase inhibitor	Concentration	Class	Inhibitory activity
Pefabloc	4.0 mM	Serine proteinases	Complete[a]
Aprotinin	0.3 μM	Serine proteinases	Complete
Soybean trypsin inhibitor	2.0 mM	Serine proteinases	Complete
Benzamidine	1–10 mM	Serine proteinases	Weak
Elastatinal	50–100 μM	Elastase	None
Antipain dihydrochloride	100 μM	Limited serine proteinases	None
Leupeptin	100 μM	Serine and thiol proteinases	None
Chymostatin	100 μM	Chymotrypsin	None
Bestatin	10 μM	Aminopeptidases	Weak
E-64	10 μM	Cysteine proteinases	None
Pepstatin	1.0 μM	Aspartic proteinases	None
EDTA	1–10 mM	Metalloproteinases	None
1,10-Phenanthroline	10 μM	Metalloproteinases	None
Phosphoramidon	100 μM	Metalloproteinases	None

[a] Complete, no immunoreactive angiostatin bands; weak, faint angiostatin bands; none, full generation of angiostatin.

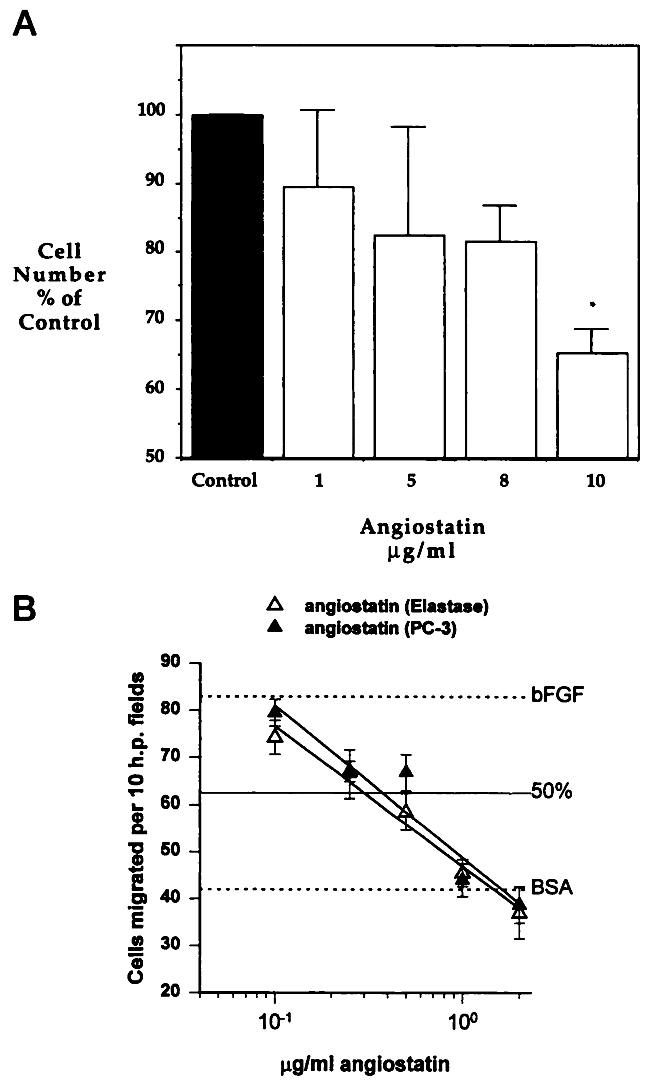

Fig. 2. PC-3-derived angiostatin inhibits endothelial cell proliferation and migration. *A*, proliferation: HUVECs were plated in growth medium and incubated overnight at 37°C. Fresh HUVEC growth medium was then supplemented with PC-3-derived angiostatin. Cells were grown for 72 h, and then an absorbance reading reflecting the number of proliferating cells was obtained. The PC-3-derived angiostatin caused a concentration-dependent decrease in the proliferation of HUVEC, with significant inhibition obtained at 10 μg/ml (*, $P < 0.05$). *Columns*, mean of samples in triplicate; *bars*, SD. *B*, bFGF-induced migration: PC-3-derived angiostatin was tested for its ability to inhibit bFGF-induced migration of bovine capillary endothelial cells in a modified Boyden chamber. A concentration-dependent inhibition of migration toward bFGF was observed with the PC-3-derived angiostatin, indistinguishable from the elastase-generated angiostatin. Background migration without the inducer in 0.1% BSA and migration in the presence of stimulatory bFGF alone are indicated. Toxicity was measured in parallel by trypan blue exclusion and was <10% at all concentrations.

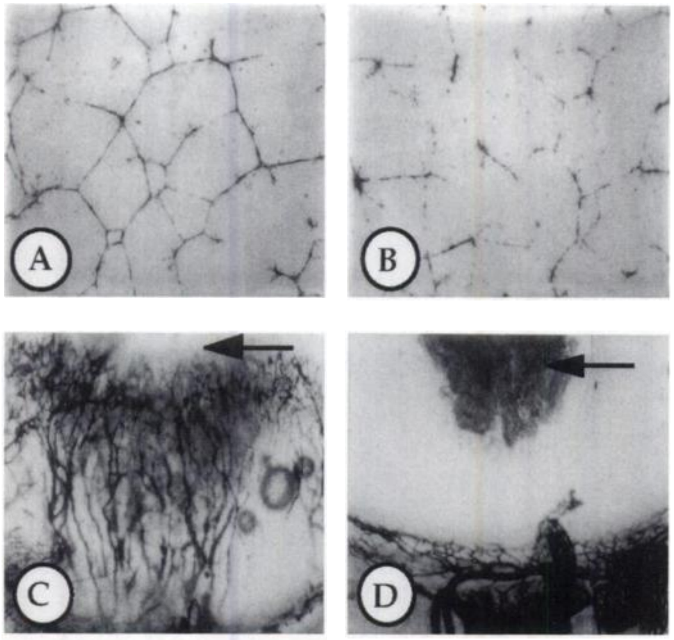

Fig. 3. PC-3-derived angiostatin inhibits human endothelial cell tube formation and bFGF-induced corneal angiogenesis. HUVECs were plated on gels of Matrigel in 24-well dishes and then were treated with 15 μg/ml of PC-3-derived angiostatin in nonconditioned RPMI. *A*, control HUVECs form branching, interconnecting networks. In contrast, PC-3-derived angiostatin caused a significant disruption of the tube network (*B*). *B*, inhibition of angiogenesis *in vivo* by PC-3-derived angiostatin. *C*, hydron pellet (*arrow*) containing bFGF induced a positive neovascular response 7 days after implantation. *D*, in contrast, no vessels are observed approaching the hydron pellet containing bFGF and 10 μg/ml PC-3-derived angiostatin (*arrow*).

minogen-angiostatin converting enzyme. The angiostatin generated by the PC-3 human prostate carcinoma line was characterized by affinity purification, Western blot, and the inhibition of many of the steps critical for angiogenesis, including endothelial cell proliferation, migration, and tube formation. In addition, the PC-3-derived angiostatin completely inhibited bFGF-induced angiogenesis *in vivo*.

The PC-3 system described here appears to be a human counterpart of the angiostatin-generating Lewis lung carcinoma of the mouse (1). PC-3 cells are inhibited by angiostatin *in vivo* (3) and show tumor-dependent suppression of micrometastases (4, 15, 16). Our data suggest that the angiostatin produced *in vivo* by the enzyme activity elaborated by the PC-3 tumor cells may be responsible for this suppression. In patients, it is possible that the expression of plasminogen-angiostatin converting activity and the generation of angiostatin could offer one compelling explanation for the indolent course of human primary prostatic carcinoma (17) and the relatively slow rate of development of clinically detectable metastases in many patients (18).

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