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


Division of Hematology/Oncology [S. G., P. T., M. P., L. R., D. L. C., G. A. S.], Department of Veterans Affairs Lakeside Medical Center [H. C. K.], Department of Obstetrics and Gynecology [M. S. S.], Department of Pediatrics, Children's Memorial Hospital [H. W. S.], Division of Endocrinology, Metabolism, and Molecular Medicine [L. M.], and Department of Microbiology-Immunology and R. H. Lurie Cancer Center [O. V., N. B.], Northwestern University School of Medicine, Chicago, Illinois 60611, and Department of Pathology [J. E.], Duke University, Durham, North Carolina 27710

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 kringle 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 bFGF1-induced corneal angiogenesis is inhibited in mice bearing s.c. PC-3 tumors (5) suggests that the antiangiogenic factor is an angiogenesis inhibitor. We now report that PC-3 cells secrete enzymatic activity able to cleave plasminogen to a 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% CO₂. 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 (572624; 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 (Mobilinob, 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-pNA; substrate III, pGlu-Pro-Val-pNA; substrate IV, Suc-Ala-Ala-Pro-Aba-pNA; 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, equilibrated 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 mM e-aminoacrylic 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 (A280) of 8.0 (2). The purified angiostatin was also examined by Coomassie Brilliant Blue staining of polyacrylamide gels and immunodetection by Western blot.

Received 8/22/96; accepted 9/18/96.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work supported in part by The Feinberg Cardiovascular Research Institute, a grant-in-aid from the American Cancer Society, IL Division (to G. A. S.), Veterans Administration Merit Review Research Grant (H. C. K.), and NIH Grants CA58900 (M. S. S.) and CA52750 and CA64239 (N. B.).

2 To whom requests for reprints should be addressed, at Division of Hematology/ Oncology, 303 East Chicago Avenue, Searle Building Suite 3—565, Chicago, Illinois, 60611.

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 NH₂-terminus of the angiostatin bands, 10 μg of the affinity purified PC-3-derived angiostatin was electrophoresed on a 12% SDS-polyacrylamide gel, electrophilically transferred to a polyvinylidene 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 plate (Nunc, Roskilde, Denmark) and incubated at a concentration of 5.0 × 10⁴ 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 μg/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 10⁵ cells/ml on the lower surface of a gelatinized membrane (Nuclepore Corp., Pleasanton, CA) in an inverted Boyden chamber, and incubated for 1.5–2 h to allow cell attachment. The chambers were reinserted, 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 1.0 × 10⁵ 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 18–18 h at 37°C in a 5% CO₂ 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. 1A), similar to those observed by O’Reilly et al. (1). Examination of SFCM from two additional human prostate carcinoma cell lines, DU-145 and LnCaP, 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. 1B). Dilution of the PC-3 SFCM resulted in a proportional decrease in angiostatin generation (Fig. 1C). 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. 1A). 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...
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 elastatin, 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. 1D). The amino-terminal sequence of all three bands was KYYLSECKTG, 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. 2A). PC-3-derived angiostatin also inhibited the bFGF-induced migration of bovine capillary endothelial cells (Fig. 2B) with an ED₅₀ 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. 3C). In contrast, angiostatin at 10 μg/ml completely inhibited the bFGF-induced angiogenic response in three of three animals (Fig. 3D). 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.

**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.

<table>
<thead>
<tr>
<th>Proteinase inhibitor</th>
<th>Concentration</th>
<th>Class</th>
<th>Inhibitory activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pefabloc</td>
<td>4.0 mm</td>
<td>Serine proteinases</td>
<td>Complete*</td>
</tr>
<tr>
<td>Aprotinin</td>
<td>0.3 mm</td>
<td>Complete</td>
<td></td>
</tr>
<tr>
<td>Soybean trypsin inhibitor</td>
<td>2.0 mm</td>
<td>Complete</td>
<td></td>
</tr>
<tr>
<td>Benzamidine</td>
<td>1–10 mm</td>
<td>Complete</td>
<td></td>
</tr>
<tr>
<td>Elastatin</td>
<td>50–100 μm</td>
<td>Elastase</td>
<td>None</td>
</tr>
<tr>
<td>Antipain dihydrochloride</td>
<td>100 μm</td>
<td>Limited serine proteinases</td>
<td>None</td>
</tr>
<tr>
<td>Leupeptin</td>
<td>100 μm</td>
<td>Serine and thiol proteinases</td>
<td>None</td>
</tr>
<tr>
<td>Chymostatin</td>
<td>100 μm</td>
<td>Chymotrypsin</td>
<td>None</td>
</tr>
<tr>
<td>Bestatin</td>
<td>10 μM</td>
<td>Aminopeptidases</td>
<td>Weak</td>
</tr>
<tr>
<td>E-64</td>
<td>10 μM</td>
<td>Cysteine proteinases</td>
<td>None</td>
</tr>
<tr>
<td>Pepstatin</td>
<td>10 μM</td>
<td>Atripic proteinases</td>
<td>None</td>
</tr>
<tr>
<td>EDTA</td>
<td>1–10 mm</td>
<td>Metalloproteinases</td>
<td>None</td>
</tr>
<tr>
<td>1,10-Phenanthroline</td>
<td>10 μM</td>
<td>Metalloproteinases</td>
<td>None</td>
</tr>
<tr>
<td>Phosphoramidon</td>
<td>100 μM</td>
<td>Metalloproteinases</td>
<td>None</td>
</tr>
</tbody>
</table>

* Complete, no immunoreactive angiostatin bands; weak, faint angiostatin bands; none, full generation of angiostatin.
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).

Acknowledgments

We thank Ivy Weiss, Owen Schnaper, and Ryan Schultz for expert technical assistance and M. S. O'Reilly for providing the purified angiostatin.

References

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