Angiostatin Generation by Cathepsin D Secreted by Human Prostate Carcinoma Cells*

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Angiostatin, a potent endogenous inhibitor of angiogenesis, is generated by cancer-mediated proteolysis of plasminogen. The culture medium of human prostate carcinoma cells, when incubated with plasminogen at a variety of pH values, generated angiostatic peptides and miniplasminogen. The enzyme(s) responsible for this reaction was purified and identified as procathepsin D. The purified procathepsin D, as well as cathepsin D, generated two angiostatic peptides having the same NH2-terminal amino acid sequences and comprising kringles 1–4 of plasminogen in the pH range of 3.0–6.8, most strongly at pH 4.0 in vitro. This reaction required the concomitant conversion of procathepsin D to catalytically active pseudocathepsin D. The conversion of pseudocathepsin D to the mature cathepsin D was not observed by the prolonged incubation. The affinity-purified angiostatic peptides inhibited angiogenesis both in vitro and in vivo. Importantly, procathepsin D secreted by human breast carcinoma cells showed a significantly lower angiostatin-generating activity than that by human prostate carcinoma cells. Since deglycosylated procathepsin D from both prostate and breast carcinoma cells exhibited a similar low angiostatin-generating activity than that by human prostate carcinoma cells, since deglycosylated procathepsin D from both prostate and breast carcinoma cells exhibited a similar low angiostatin-generating activity than that by human prostate carcinoma cells. The seminal vesicle fluid from patients with prostate carcinoma contained the mature cathepsin D and procathepsin D, but not pseudocathepsin D, suggesting that pseudocathepsin D is not a normal intermediate of procathepsin D processing in vivo. The present study provides evidence for the first time that cathepsin D secreted by human prostate carcinoma cells is responsible for angiostatin generation, thereby causing the prevention of tumor growth and angiogenesis-dependent growth of metastases.

Angiogenesis is an important process of new microblood vessel formation under both physiological and pathological conditions. Because angiogenesis is essential for tumor growth and metastases, the discovery of angiogenesis inhibitors is considered to represent a potential approach to treat cancer. It has so far been suggested that the metastatic growth of carcinoma cells is associated with induced angiogenesis and that the rate of tumor growth correlates with the formation of new blood vessels (1). Higher vascular tumors can be stimulated by angiogenic growth factors released by tumors and host tissues, thereby producing metastases at a higher rate than less angiogenic tumors. On the other hand, it has been known that certain types of primary tumors, such as murine Lewis lung carcinoma and human prostate carcinoma cell lines (PC-3), can produce angiogenic inhibitors in order to suppress the tumor growth at metastatic sites, besides angiogenic growth factors (2, 3). Therefore, the growth rate of a tumor may be the result of the balance between positive and negative angiogenic effects.

Angiostatin and endostatin are endogenous angiogenic inhibitors produced by proteolysis of plasminogen and collagen XVIII, respectively (1, 4). Considering the generation of these angiogenic inhibitors, it is most likely that carcinoma cells produce proteinase(s) generating angiogenic inhibitors. Angiostatin was originally discovered in serum and urine as an endogenous inhibitor of angiogenesis and endothelial cell proliferation (2, 3) and comprised the first four kringle domains of plasminogen. This fragment inhibits endothelial cell proliferation in vitro (2, 5–7) and the basic fibroblast growth factor (bFGF)1-induced angiogenesis in mouse cornea and lung metastasis of Lewis lung carcinoma in mice in vivo (2, 3). Although the mechanism of angiostatin formation in vivo, as well as the molecular mechanism of angiostatin action, remains unknown, recent in vitro evidence has suggested that this protein is generated by multiple enzymatic actions. These include matrix metalloproteinase (MMP)-2 (8), MMP-3 (9), MMP-7 (10), MMP-9 (10), MMP-12 (11), plasmin (12, 13), and tumor cell-derived plasmin thiolreductase (13, 14).

Recently, human prostate carcinoma cells (PC-3) have been shown to express an enzymatic activity in the serum-free conditioned medium that can generate bioactive angiostatin from plasminogen (12), in which plasminogen is suggested to be initially converted to proangiogenic plasmin by cleavage of the Arg560–Val561 bond by plasminogen activators such as urokinase- and tissue-type plasminogen activators and then converted to angiostatin by autoproteolysis of plasmin in the presence of free sulphydryl donors. The importance of plasmin in cancer-mediated conversion of plasminogen to angiostatin was also described in human fibrosarcoma cells (HT1080) (14).

1 The abbreviations used are: bFGF, basic fibroblast growth factor; FCS, fetal calf serum; LBS I, plasminogen lysine binding site I; MMP, matrix metalloproteinase; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline.

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However, it remains to be determined whether angiostatin production by prostate carcinoma cells is totally dependent on the plasmin action.

Here we report that procathepsin D produced directly by human prostate carcinoma cells is responsible for the generation of angiostatic peptides comprising kringle domains 1–4 of plasminogen and that the isolated angiostatic peptides can inhibit endothelial cell proliferation in vitro and bFGF-induced angiogenesis in vivo. In addition, we address the question about the difference of procathepsin D molecules between human prostate and breast carcinoma cells in the ability of angiostatin generation.

**EXPERIMENTAL PROCEDURES**

**Materials**—Human cathepsin D and pepsin were purchased from Sigma. Porcine pancreatic elastase was from Elastin Product Company, Inc. A chromogenic substrate (S-2251) was from Chromogenix AB, Sweden. Lysine-Sepharose 4B, Sephadex G-75, Superdex G-200, Phenyl-Sepharose HP, and hydroxyapatite were from Amersham Pharma Biotech. Angiostatin consisting of the first three kringle domains of plasminogen was from TechnoClone GmbH (Vienna, Austria). Human cathepsin E was prepared from human erythrocyte membranes as described previously (15). Polyclonal antibodies against human cathepsin D and human plasminogen were from Calbiochem-Boehringer. Benzamidine-HCl was from Dainippon Pharmaceutical Co. (Tokyo). Human umbilical endothelial cells were from Clonetics Corp. Seminal vesicle fluid was obtained from two patients with prostate carcinoma in the Department of Urology, Kyushu University Hospital (Fukuoka, Japan), under an Institutional Review Board-approved protocol with subjects providing informed consent.

**Cell Culture**—PC-3 cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), penicillin G (100 units/ml), and streptomycin (100 μg/ml). MCF-7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FCS, 1% nonessential amino acids, 1 mM sodium pyruvate, and the antibiotics. Human omental microvascular endothelial cells that were isolated from normal omental tissue removed during surgery as described previously (16) were cultured in M-199 medium containing 10% FCS. Human prostate carcinoma (PC-3) cells and human breast carcinoma (MCF-7) cells were obtained from Dainippon Pharmaceutical Co. (Tokyo). Human umbilical endothelial cells were from Clonetic Corp. Seminal vesicle fluid was obtained from two patients with prostate carcinoma in the Department of Urology, Kyushu University Hospital (Fukuoka, Japan), under an Institutional Review Board-approved protocol with subjects providing informed consent.

**Isolation of Plasminogen and Its Related Fragments**—Human plasminogen and its related fragments were purified from the pooled human plasma in the presence of umbilical endothelial cells were cultured as recommended by the supplier.

**EXPERIMENTAL PROCEDURES**

**Isolation of Plasminogen and Its Related Fragments**—Human plasminogen and its related fragments were purified from the pooled human plasma by hydroxyapatite chromatography (1 mg/ml), followed by gel filtration on Sephacryl S-200 (2.5 × 30 cm) equilibrated with 10 mM triethanolamine–HCl, pH 7.4, containing 25 mM NaCl (buffer A), and concentrated by ultrafiltration on Amicon Diaflo YM10 membrane and then subjected to Superdex G-200 (1.0 × 60 cm) chromatography equilibrated with buffer A. The elution was set at the position corresponding to an apparent molecular mass of 50–60 kDa. After concentration and dialysis against buffer A, the active fraction was subjected to a Q-Sepharose HP column (0.5 × 6.5 cm) equilibrated with buffer A. The enzyme activity was eluted by the gradient of NaCl ranging from 0 to 1.0 M. The active fractions were pooled and dialyzed against 20 mM sodium phosphate buffer, pH 7.2, and then subjected to a hydroxyapatite column (1.0 × 10 cm) equilibrated with 20 mM sodium phosphate buffer. The active fractions were then subjected to the gradient of sodium phosphate ranging from 20 to 200 mM. The enzyme responsible for the generation of angiostatin was eluted at a concentration of about 50 mM sodium phosphate.

**Purification of Angiostatic Peptides**—The angiostatic peptides were purified from human plasminogen fragments hydrolyzed by the culture medium from PC-3 cells. Human plasminogen (25 mg/ml) was incubated with the culture medium from PC-3 cells (0.8 mg/ml) in 100 mM citrate/phosphate buffer, pH 4.0, at 37 °C overnight. Then the mixture was subjected to affinity chromatography on a lysine-Sepharose 4B column equilibrated with 50 mM Tris-HCl, pH 7.4, containing 0.15 M NaCl and 5 mM EDTA. Angiostatic peptides consisting of the first four kringle domains of plasminogen were eluted with 0.1 M e-aminoacrid acid in the same buffer. The peptides were further purified by gel filtration on a Sephadex G-75 column. The main peak fractions were pooled, dialyzed against PBS, and stored at −80 °C until use.

**Assay Procedures**—The angiostatin-generating activity was assayed by enzyme-linked immunosorbent assay with two types of antibodies against human plasminogen: the antibodies to human plasminogen and the monoclonal antibody against LBS I. Angiostatin-generating activity was evaluated by studying remaining plasminogen and the difference of procathepsin D molecules between human prostate and breast carcinoma cells in the ability of angiostatin generation for a certain period. Microwells were coated with 150 μg of 50 mM carbonate-buffer, pH 9.6, containing the monoclonal antibody to miniplasminogen (5 mg/ml) at 4 °C overnight. Unbound antibody was drained off, and the wells were blocked with 300 μl of bovine serum albumin (5 mg/ml) in the coating buffer for 1 h at room temperature. The wells were washed four times with PBS containing 0.05% Tween 20. Meanwhile, samples (50 μl) were mixed with 50 μl of plasminogen (1.0 mg/ml), 100 μl of citrate/phosphate buffer (pH 4.0) and incubated at 37 °C for 1 h. The reaction mixture was then diluted 2500-fold with PBS containing aprotinin (10 units/ml), 1% bovine serum albumin, and 0.05% Tween 20. The diluted sample (100 μl) was added to the coated wells and incubated at 37 °C for 1 h. After washing, 50 μl of horseradish peroxidase-conjugated anti-human LBS I antibody (20 ng/ml) was added to the wells and incubated at 37 °C for 1 h. After washing, 100 μl of freshly prepared o-phenylenediamine solution was added and allowed to stand in the dark at room temperature for 20 min. The reaction was stopped by adding 4 N sulfuric acid. The absorbance at 490 nm was determined promptly. 1 unit was defined as the enzyme activity that degrades 20 μg of plasminogen for 1 h at 37 °C.

**Plasmin activity was assayed by the method of Friberger et al. (19). Briefly, samples were mixed with 50 μl of urokinase-type plasminogen activator (20 units/ml) in 50 mM Tris-HCl, pH 7.4, containing 10 mM NaCl. After a 15-min incubation at 37 °C, 50 μl of the chromogenic substrate S-2251 was added to the mixture and incubated at 37 °C for 1 h. After washing, 50 μl of horseradish peroxidase-conjugated goat anti-rabbit IgG or rabbit anti-mouse IgG antibody (diluted 1:1000–1:4000) were added to the wells and incubated at 37 °C for 1 h. After washing, the membranes were incubated for 1 h with the secondary antibody solution containing horseradish peroxidase-conjugated goat anti-rabbit IgG or rabbit anti-mouse IgG antibody (diluted 1:1000–1:4000). Enhanced chemiluminescence (PerkinElmer Life Sciences) was used for visualization.

**Immunoblotting**—Proteins were electrophoresed on polyvinylidene difluoride filters. After blocking in 4% skim milk in 10 mM Tris-HCl and 0.15 M NaCl at pH 7.4 containing 0.1% Tween 20 for 2 h at room temperature, the membranes were incubated in 10 mM Tris-HCl, pH 7.4, containing the first antibodies (10 μg/ml). After washing, the membranes were incubated for 1 h with the secondary antibody solution containing horseradish peroxidase-conjugated goat anti-rabbit IgG or rabbit anti-mouse IgG antibody (diluted 1:1000–1:4000). Enhanced chemiluminescence (PerkinElmer Life Sciences) was used for visualization.

**Treatment of Plasminogen with Various Aspartic Proteinases**—Plasminogen (250 μg/ml) was treated with cathepsin D (5 μg/ml), cathepsin E (5 μg/ml), pepsin (5 μg/ml), or carboxypeptidase E (5 μg/ml) in citrate/phosphate buffer at pH 4.0. Each digest was sampled at appropriate time intervals and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (12.5% polyacrylamide, and 2.5 μg of protein/lane).

**NH2-Terminal Amino Acid Sequence Analyses**—The proteins that had been separated by SDS-PAGE were electrophoretically transferred from the gels onto polyvinylidene difluoride membranes. The protein bands on the membranes were stained with Coomassie Brilliant Blue R-250, excised, and subjected to the NH2-terminal amino acid sequence analysis by an automatic protein/peptide sequencer (Applied Biosystems model 477A).
Angiostatin Generation by Secreted Cathepsin D

Endothelial Proliferation Assay—The proliferation assay of human omentum microvascular endothelial cells was performed by the method of O’Reilly et al. (2), with a slight modification. Briefly, the cells (5000 cells) were plated onto gelatinized culture plates (24 wells) and incubated in M199 containing 10% FCS (24 wells) and incubated in M199 containing 10% FCS, penicillin G (100 units/ml), streptomycin (100 µg/ml), and amphotericin B (1 µg/ml) for 72 h. After treatment with trypsin-EDTA, the dispersed cells were counted using a Coulter counter.

RESULTS

Angiostatin Generation from Human Plasminogen by Secreted Proteinases by Human Prostate Carcinoma Cells—The culture medium from PC-3 cells was incubated with plasminogen at pH 4.0 and 37 °C overnight in the presence of various protease inhibitors. The remaining angiostatin-generating activity was determined by enzyme-linked immunosorbent assay as described under “Experimental Procedures.” The values are expressed as percentages of the activity determined in the absence of proteinase inhibitors and are means of three determinations. E-64, L-trans-epoxysuccinyl-leucyl-aminodipetyl chloromethyl ketone.

| Inhibitor     | Concentration | Class          | Activity remaining % |
|--------------|---------------|----------------|----------------------|
| None         | 10 µM         | Cysteine proteinases | 100                  |
| E-64         | 1 mg/l        | Metalloproteinases | 98                   |
| Pepstatin A  | 1 µM          | Aspartic proteinases| 0                    |
| Elastatinal   | 50 µM         | Elastase        | 97                   |
| Benazanide    | 10 mg/l       | Serine proteinases| 98                   |
| Aprotinin     | 1 mg/l        | Serine proteinases| 100                  |

Fig. 1. Effect of pH on the degradation of plasminogen by the culture medium of human prostate carcinoma (PC-3) cells. A, the culture medium of PC-3 cells (10 µg) was incubated at 37 °C overnight with human plasminogen (25 µg of protein) at various pH values in citrate/phosphate buffer (100 mM) followed by SDS-PAGE under nonreducing conditions. Lane C, untreated human plasminogen. B, analyses of plasminogen degradation products generated at pH 4.0 by SDS-PAGE and immunoblotting. After electrophoresis, the proteins were visualized by Coomassie Brilliant Blue staining (lane 1) followed by immunoblotting with the monoclonal antibody to miniplasminogen (lane 2) and antibodies to LBS 1 (lane 3).
Characterization of the Process of Angiostatin Generation by Procathepsin D—The time-dependent profile for the generation of angiostatic peptides from plasminogen was determined by a proteolytic cleavage of 41-kDa protein was generated by a proteolytic cleavage of procathepsin D to the 41-kDa form (Fig. 3A). The time-dependent conversion of procathepsin D to pseudocathepsin D concomitant with the generation of angiostatic peptides. The purified procathepsin D (0.25 μg) was incubated with human plasminogen (25 μg) at pH 4.0 and 37 °C. Aliquots were withdrawn from the reaction mixture at the indicated times and subjected to SDS-PAGE under nonreducing conditions (B) followed by immunoblotting with antibodies to human cathepsin D (A).

Angiostatin Generation Requires Conversion of Procathepsin D to Pseudocathepsin D—Angiostatic peptides and miniplasminogen were generated time-dependently by incubation of plasminogen with procathepsin D at pH 4.0 and 37 °C for up to 1 h (Fig. 3B). The 45-, 42-, and 35-kDa peptides all were resistant to further degradation by prolonged incubation up to 4 h (not shown). These results were consistent with those obtained with the culture medium of PC-3 cells. The generation of these peptides was in fair agreement with the conversion of procathepsin D to the 41-kDa form (Fig. 3A). Within a 15-min incubation, about 50% of procathepsin D was converted to the 41-kDa form. The complete conversion was accomplished within a 30-min incubation. The NH₂-terminal amino acid sequence of the 41-kDa protein was found to be IAKGP, which corresponded to the NH₂-terminal amino acid sequence of the predicted amino acid sequence of plasminogen. The results indicated that the 40-kDa peptide was converted to the 32-kDa peptide was identical with that of the 40-kDa peptide. The NH₂-terminal amino acid sequence of the 32-kDa peptide was identified as the amount of enzyme required to degrade 20 μg of plasminogen at 37 °C for 1 h.

The angiostatin-generating activity was determined by enzyme-linked immunosorbent assay as described under "Experimental Procedures."
Angiostatic peptides generated by pseudocathepsin D in a dose-dependent manner. The angiogenesis was completely inhibited by 50 ng of angiostatic peptides. Similarly to pseudocathepsin D and cathepsin D, both cathepsin E and pepsin efficiently generated the angiostatic peptides. However, cathepsin E and pepsin further degraded the angiostatic peptides into smaller fragments by prolonged incubation up to 4 h, whereas cathepsin D, as well as pseudocathepsin D, did not cause the degradation of angiostatic peptides for 4 h of incubation.

Inactivation of Plasmin by Pseudocathepsin D—Plasmin is known to play a key role in producing angiogenic factors such as MMPs and cytokines (22). Cancer invasion is thought to be initiated by the activation of plasminogen and procollagenase cascade (23, 24). In addition, plasmin has been shown to be important in cancer-mediated conversion of plasminogen to angiostatin (14, 22, 25). These results thus suggest that inactivation of plasmin modulates angiogenesis in tumors. If the Glu^Glu--Ala^Ala^Ala^Ala^Ala bond in the serine protease domain of plasminogen is cleaved by pseudocathepsin D, the plasminogen-plasmin converting activity may be lost. To examine this possibility more directly, the plasmin activity was determined by use of plasminogen activator after incubation of plasminogen with procathepsin D at pH 4.0 for 37 °C (Fig. 7). The plasmin activity was lost time-dependently, indicating that angiostatin generation by pseudocathepsin D results in the concomitant loss of plasminogen-plasmin converting activity.

Inhibition of Human Endothelial Cell Proliferation in Vitro and b-FGF-induced Angiogenesis in Mouse Cornea in Vivo by Purified Angiostatic Peptides—Angiostatic peptides generated by pseudocathepsin D were purified from procathepsin D-treated plasminogen by use of lysine-Sepharose and Sephadex G-75 columns. The NH₂-terminal amino acid sequence analyses revealed that purified angiostatic peptides had the same NH₂-terminal amino acid sequences and consisted of kringle 1–4 of plasminogen, suggesting that the difference in their apparent molecular masses in an SDS gel is due to the heterogeneity in the carbohydrate modification. The proliferation of human microvascular endothelial cells was inhibited by these peptides in a dose-dependent manner (Fig. 8A). A similar result was obtained by angiostatin generated by elastase, which was known to consist of kringle 1–3 of plasminogen.

The effect of the purified angiostatic peptides on b-FGF-induced angiogenesis in mouse cornea was also investigated (Fig. 8B). Hydrone pellets containing 50 ng of b-FGF with or without 50 ng of angiostatic peptides were implanted into the cornea of three mice. 6 days after implantation, b-FGF alone clearly induced angiogenesis in corneas, whereas b-FGF containing 100 ng of angiostatic peptides did not induce the increased angiogenesis. The b-FGF-induced angiogenesis was inhibited by angiostatic peptides generated by pseudocathepsin D in a dose-dependent manner. The angiogenesis was completely inhibited by 100 ng of angiostatic peptides.

Comparison of Procathepsin D Molecules Secreted by Human Prostate and Breast Carcinoma Cells—It is well known that procathepsin D is abundantly released by human breast carcinoma cells (26–29). We analyzed whether the culture medium of human breast carcinoma cells (MCF7) could exhibit the angiostatin-generating aspartic proteinase activity found in PC-3 cells. Surprisingly, the angiostatin-generating activity was barely detectable in the culture medium from MCF7 cells, similarly to the control medium from human umbilical vascular endothelial cells (Fig. 9). To determine whether this was due to the inability of procathepsin D secreted by MCF7 cells to generate angiostatic peptides, procathepsin D was also purified from the culture medium of MCF7 cells. The final preparation of procathepsin D gave a single protein band by SDS-PAGE, but its mobility was slightly faster than that from PC-3 cells (Fig. 10A). After N-glycosidase F treatment, both procathepsin

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**FIG. 4. Characterization of time-dependent generation of angiostatic peptides from plasminogen by pseudocathepsin D.** Plasminogen (25 μg) and procathepsin D (0.1 μg) were incubated at pH 4.0 and 37 °C. Aliquots were withdrawn from the reaction mixture at the indicated times and subjected to SDS-PAGE under nonreducing (A) and reducing conditions (B). C, a schematic model for the generation of angiostatic peptides from plasminogen by pseudocathepsin D. The cleavage of plasminogen by pseudocathepsin D, as indicated by the arrows, first occurs at the Leu^Pro^Pro bond, followed by the Leu^Phe^Phe bond. Then an additional cleavage occurs at the Glu^Phe^Phe bond. *+, an N-linked glycosylation site; 0, an O-linked glycosylation site.

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fragment by cleavage of the Glu^Glu--Ala^Ala bond. Based on these data, the sequential cleavage sites of plasminogen by pseudocathepsin D were summarized in Fig. 4C. Plasminogen appears to be cleaved by pseudocathepsin D at the Leu^Pro^Pro bond followed by cleavage at the Leu^Phe^Phe bond and finally at the Glu^Ala--Ala bond.

When plasminogen was incubated with procathepsin D at various enzyme/substrate ratios, the 55- and 52-kDa angiostatic peptides and the 32-kDa peptide were rapidly generated at an enzyme/substrate ratio below 1:20 (Fig. 5A). At ratios between 1:40 and 1:100, the 40-kDa intermediate was produced prior to the 32-kDa peptide formation, besides the angiostatic peptides. The effect of pH on the generation of angiostatic peptides by procathepsin D was examined at the enzyme/substrate ratio of 1:20 (Fig. 5B) and 1:10 (Fig. 5C). The efficient angiostatin generation was observed at pH values between 3.5 and 5.5 (Fig. 5B). Although angiostatin generation was relatively slow at pH values above 6.0, angiostatic peptides, especially the 55-kDa peptides, were clearly produced by this enzyme even at pH 6.8 (Fig. 5C). Therefore, we concluded that pseudocathepsin D produced by procathepsin D autoactivation could generate angiostatic peptides at a wide range of pH values between 3.0 and 6.8.

**Generation of Angiostatic Peptides by Cathepsin E and Pepsin—**To determine whether other relevant aspartic proteinases have the ability to generate angiostatic peptides, plasminogen was treated with human cathepsin E and pepsin, as well as cathepsin D, under the same conditions as procathepsin D (Fig.
D molecules showed the same mobility, suggesting that both enzymes are different in carbohydrate structures.

When MCF7-derived procathepsin D was incubated with plasminogen at pH 4.0 and 37 °C, angiostatic peptides were scarcely generated within 12 h incubation (Fig. 10B). Only a small amount of angiostatic peptides was produced by this enzyme after 24 h of incubation. Interestingly, N-glycosidase F-treated procathepsin D from PC-3 cells showed a marked decrease in the angiostatin-generating activity. These data suggest that the structural difference of procathepsin D molecules is associated with their ability to generate angiostatic peptides.

Angiostatin could be generated by procathepsin D secreted by human prostate carcinoma PC-3 cells even at pH values around neutrality (Fig. 5, B and C). However, it remains to be answered whether procathepsin D is secreted extracellularly in vivo in patients with prostate carcinoma. We thus examined if procathepsin D was detected in the seminal fluid from patients with prostate carcinoma. Although PC-3 cells secreted procathepsin D alone in the culture medium in vitro, the seminal vesicle fluid from patients with prostate carcinoma contained not only procathepsin D but also cathepsin D (Fig. 11). When treated at pH 4.0 and 37 °C for 1 h, procathepsin D from PC-3 cells and the seminal vesicle fluid completely converted to pseudocathepsin D. The molecular mass of cathepsin D was not changed by the treatment. These results suggest that angiostatic peptides may be generated in vivo by both cathepsin D and procathepsin D in patients with prostate carcinoma.

**DISCUSSION**

This study provides evidence that cathepsin D and/or pseudocathepsin D are responsible for the angiostatin-generating activity secreted by human prostate carcinoma cells. In vitro studies showed that procathepsin D purified from the culture medium of PC-3 cell could generate angiostatic peptides from plasminogen in a wide range of pH values between 3.0 and 6.8 (Fig. 5). The most efficient generation of angiostatic peptides by procathepsin D was observed at pH 4.0–5.0, and this process required the concomitant conversion of procathepsin D.
sin D to catalytically active pseudocathepsin D. At pH values below 3.0 or above 6.0, however, the rate of plasminogen cleavage by the enzyme was less than 20% of that at pH 4.0–5.0. Angiostatin generation was strongly inhibited by pepstatin A at each pH value, indicating that the potency of angiostatin generation is dependent on the rate of the conversion of procathepsin D to pseudocathepsin D. The cleavage site at the Leu46–Ile47 bond in the procathepsin D resulted in a loss of 3 kDa. Pseudocathepsin D described here appeared to correspond to the catalytically active intermediate formed when procathepsin D from recombinant (30) and natural sources (31–35) is exposed to acid pH. However, pseudocathepsin D was not converted to the mature cathepsin D even by the prolonged incubation. In contrast, the seminal vesicle fluid from patients with prostate carcinoma contained the mature cathepsin D and procathepsin D and did not show pseudocathepsin D (Fig. 11). These results are consistent with the previous findings that the autoproteolysis of procathepsin D alone cannot generate the mature enzyme found in vivo (35) and that pseudocathepsin D is not a normal intermediate of procathepsin D processing in vivo (36). Taken together, the present results suggest that the conversion of procathepsin D to the mature enzyme via pseudocathepsin D in vivo may occur more rapidly as compared with the reaction under in vitro conditions and that the conversion of pseudocathepsin D to cathepsin D may be mediated by other enzyme(s).

It is well known that the extracellular pH of tumors is acidic, although the precise mechanism for the formation and maintenance of the acidic extracellular pH remains unknown. However, recent evidence has indicated that the proton pump of the vacuolar H⁺-ATPase at the plasma membrane of cancer cells contributes to the formation and maintenance of acidic pH in the extracellular space of tumors (37). This enzyme normally resides in acidic organelles such as endosomes and lysosomes and contributes to the maintenance of acidic environments. In the actively bone-resorbing osteoclasts, this enzyme resides at the plasma membrane of the ruffled border and works to form and maintain an acid environment in the resorption lacuna (38–41). Therefore, when located at the plasma membrane of the cancer cells, this enzyme may work to extrude acid extracellularly. In addition, anoxia may contribute to maintain an acid environment of tumors (42). Negatively charged macromolecules, such as sialoglycoproteins and mucopolysaccharides, on the plasma membrane of cancer cells are also associated with the formation and maintenance of an acid environment of tumors. Furthermore, the β subunit of mitochondrial ATP synthase (Fₒ,Fᵥ-ATPase) has been present on the surface of cancer cells (43) as well as endothelial cells (44). It is thus interesting to speculate that this enzyme at the

![Fig. 8.](image1.png)

Fig. 8. Inhibition of human endothelial cell proliferation in vitro and bFGF-induced mouse corneal neovascularization in vivo by purified angiostatic peptides. A, various concentrations of angiostatic peptides generated by pseudocathepsin D (○) and elastase (●) were incubated with human omentum microvascular endothelial cells at 37 °C for 72 h. Angiostatic peptides generated by pseudocathepsin D, which consist of kringles 1–4 of plasminogen, inhibited endothelial cell proliferation in a dose-dependent manner. A similar inhibition profile for endothelial cell proliferation was observed with angiostatic peptides generated by elastase, which consist of kringles 1–3. B, concentration-dependent inhibition of the mouse cornea neovascularization by angiostatic peptides. Hydron pellets containing 50 ng of bFGF were implanted in mouse cornea in the presence or absence of various concentrations of angiostatic peptides. The vessel lengths were determined 6 days after bFGF were implanted in mouse cornea. The error bars show the means ± S.D.

![Fig. 9.](image2.png)

Fig. 9. Comparison of secreted procathepsin D between human prostate and breast carcinoma cells. Plasminogen (25 μg) was incubated at pH 4.0 and 37 °C overnight with culture media obtained from human umbilical vascular endothelial cells, human breast carcinoma cells (MCF7), and PC-3 cells, and then the reaction products were analyzed by SDS-PAGE under nonreducing conditions (A) and immunoblotting with antibodies to human plasminogen (B). Lane 1, untreated human plasminogen; lane 2, human umbilical vascular endothelial cells; lane 3, PC-3 cells; lane 4, MCF7 cells.
plasma membrane of cancer cells also may work to produce extracellular ATP and to maintain an acidic extracellular pH. Therefore, it is most likely that cancer cells in tumors provide an acidic pH environment formed by multiple pathways in vivo that is favorable for the activation of procathepsin D, thereby generating angiostatic peptides.

Angiostatic peptides produced by pseudocathepsin D consisted of kringles 1–4 of plasminogen. Pseudocathepsin D initially cleaved at the site between Leu74 and Phe75 of plasminogen and subsequently at the site between Leu451 and Pro452. Finally, it cleaved at the site between Glu699 and Ala700. In parallel with angiostatin generation, pseudocathepsin D nicked the serine proteinase domain of plasminogen, resulting in the loss of the potency of plasminogen-plasmin converting activity (Fig. 7). This may cause loss of the angiostatin-generating activity by plasmin. On the other hand, plasmin is known to be a key factor to generate angiogenic factors (22). Therefore, inactivation of plasmin by pseudocathepsin D may also contribute to anti-angiogenesis. Human mature cathepsin D showed the same cleavage profile for plasminogen as the pseudocathepsin D. Interestingly, the relevant human aspartic proteinases cathepsin E and pepsin also degraded plasminogen with a similar susceptibility to generate angiostatic peptides. However, while the generated angiostatic peptides were resistant to proteolysis by pseudocathepsin D, they were further degraded by cathepsin E and pepsin by prolonged incubation. Consistent with these results, neither cathepsin E nor pepsin was detectable in the culture medium of PC-3.

Purified angiostatic peptides with kringles 1–4 of plasminogen inhibited the proliferation of human microvascular endothelial cells and the bFGF-induced angiogenesis in mouse cornea (Fig. 8). Since recombinant angiostatin carrying kringles 1–3 of plasminogen (Leu93–Pro386) has also been shown to inhibit the proliferation of bovine capillary endothelial cells and lung metastasis of mouse melanoma B16-BL6 (45), the inhibitory effect on the endothelial proliferation and the growth of several diverse carcinoma appears to be expressed by kringles 1–3 of plasminogen. Importantly, the antiproliferative activity of angiostatin has been reported to be exhibited by individual recombinant kringles 1, 2, and 3 (46), but not by kringle 4. In addition, kringle 5 has been shown to inhibit bFGF-induced proliferation and migration of bovine capillary endothelial cells (46, 47). These results suggest that the antiproliferative property of angiostatin is shared by various kringle-containing fragments of plasminogen. Taken together, the present data suggested that pseudocathepsin D generated angiostatic peptides with kringles 1–4 having the antiproliferative activity on endothelial cells. The antiproliferative effect of angiostatic peptides on endothelial cells appears to be associated with their ability to inhibit tumor angiogenesis.

Interestingly, procathepsin D purified from the culture medium of human breast carcinoma cells (MCF7) showed little or no activity for angiostatin generation (Fig. 10).
Angiostatin Generation by Secreted Cathepsin D

revealed that procathepsin D derived from PC-3 cells moved more slowly than that from MCF7 cells and that N-glycosidase F treatment resulted in identical mobility. In addition, procathepsin D from PC-3 cells exhibited a plasminogen-hydrolyzing activity much higher than that from MCF7 cells at pH 4.0, although no significant difference in their activities on synthetic peptide substrates was observed. These data suggest that procathepsin D derived from PC-3 cells moved in vivo. This reaction essentially required the concomitant conversion of procathepsin D to cathepsin D. Since both procathepsin D and cathepsin D were detected in the seminal vesicle fluid from patients with prostate carcinoma, these molecules are likely to play a role in angiostatin generation in vivo as well as in vitro.

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Angiostatin Generation by Cathepsin D Secreted by Human Prostate Carcinoma Cells

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