Anti-Thrombin Is Expressed in the Benign Prostatic Epithelium and in Prostate Cancer and Is Capable of Forming Complexes with Prostate-Specific Antigen and Human Glandular Kallikrein 2

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Anti-thrombin, a member of the serpin family and an inhibitor of thrombin and blood coagulation factor Xa, was recently shown to inhibit angiogenesis and tumor growth. In the present study, we examined the expression of anti-thrombin in benign and malignant prostate gland. Using immunohistochemistry, anti-thrombin was found in prostate epithelium and stroma cells. Tissue microarrays of tumors ($n = 112$) and three different prostate cancer cell lines (PC-3, LNCaP, and DU-145) were all positive for anti-thrombin. Abundant expression in a population of prostatic tumor cells was further evidenced by *in situ* hybridization experiments. The immunostaining for anti-thrombin was confined to the cytoplasm, was most intense in Gleason grade 3 tumors, and in part overlapped with that of prostate-specific antigen. Western blotting of benign and malignant tissue homogenates revealed a predominant 58-kd anti-thrombin immunoreactive component. *In vitro*, anti-thrombin formed complexes more readily with human kallikrein 2, particularly in the presence of heparin, and less efficiently with prostate-specific antigen. Both complexes could be recognized by polyclonal and monoclonal IgGs against anti-thrombin. We conclude that anti-thrombin is widely expressed in prostate cancer but is gradually lost in tumors of high Gleason grade. Anti-thrombin may act as a local anti-angiogenic factor, the effect of which is partially lost in poorly differentiated prostatic tumors. (Am J Pathol 2002; 161:2053–2063)

Anti-thrombin, a plasma proteinase inhibitor of the serpin superfamily, is a heparin-binding protein and the major plasma inhibitor of coagulating proteases, primarily thrombin and factor Xa.^{1,2} The capacity of anti-thrombin to inhibit thrombin increases up to 4000-fold by binding a specific pentasaccharide in heparin.³ In addition, anti-thrombin is also recognized as a proteinase inhibitor of

plasma kallikrein, plasmin, and trypsin.^{4–6} The molecular mass of anti-thrombin is ~58 kd and it contains 432 amino acids.^{7,8} Recently, anti-angiogenic and anti-tumor growth properties have been attributed to both the cleaved and the uncleaved latent forms of human plasma-derived anti-thrombin.^{9,10} Heat treatment, as used in the preparation of latent anti-thrombin from native anti-thrombin, generates a recently identified form of the molecule, denoted prelatent anti-thrombin.¹¹ The prelatent form of anti-thrombin with proteinase- and heparin-binding properties has also been shown to inhibit angiogenesis and tumor growth.¹¹ These findings indicate that factors participating in the blood clotting and fibrinolytic pathways are also involved in the regulation of angiogenesis.

Few reports on the local production of anti-thrombin in tumors are available in the literature. The expression of anti-thrombin was described in hepatocellular carcinoma,¹² and other tumors, including small-cell lung cancer, were shown to sequester anti-thrombin into their stroma.^{13,14} Recently, latent and cleaved anti-angiogenic anti-thrombin were isolated and characterized from an inoculum of human pancreatic cancer cells (BxPC-3) and were shown to inhibit the growth of a secondary tumor (Lewis lung carcinoma and HT 1080 fibrosarcoma) in an *in vivo* animal model.¹⁵

A decrease in plasma of anti-thrombin in cancer patients has been described.¹⁶ Honegger and co-workers¹⁷ also reported a decrease in plasma levels of anti-thrombin in patients with colon, ovary, and prostate cancer (CAP), especially in those with metastatic disease. The role of anti-thrombin in estrogen-treated CAP patients with decreased fibrinolytic activity was discussed more than 20 years ago.^{18,19} Reduced plasma anti-thrombin

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activity was reported to be associated with increased risk of thrombosis in patients with CAP.^{19,20}

Prostate-specific antigen (PSA), the most important serum marker for CAP^{21,22} is a chymotrypsin-like serine protease and a member of the glandular kallikrein family.^{23–25} PSA exists in free and complexed forms in blood, and measurements of the proportions of these forms, free to total (F/T) or complexed (C) PSA to α -1-anti-chymotrypsin, have been shown to improve the specificity of CAP diagnosis.^{26–28} Higher proportions of PSA- α -1-anti-chymotrypsin complexes are found in CAP than in benign prostatic hyperplasia (BPH), and this has become a widely used tool in tumor diagnosis.^{26,28} Another member of the glandular kallikrein family, human glandular kallikrein 2 (hK2), is a trypsin-like serine proteinase, structurally similar to PSA with 78% identity in the amino acid sequence.^{24,29} hK2 has been found to form complexes with protein C inhibitor, α -2-anti-plasmin and plasminogen activator inhibitor-1.^{30–32}

Diamandis and co-workers³³ recently described additional members of the glandular kallikrein family and discussed their possible role in carcinogenesis. Recent experimental evidence suggests that PSA acts as an endogenous anti-angiogenic protein by inhibiting endothelial cell proliferation and migration *in vitro* and endothelial cell response to the angiogenic stimulators, such as fibroblast growth factor-2 and vascular endothelial growth factor.³⁴ In a mouse model of metastatic CAP, daily injections of PSA reduced the number of lung tumor nodules by 40%.

The present study was undertaken to determine whether anti-thrombin is expressed in the benign and malignant prostate and to characterize molecular forms, as well as to further investigate its capacity to form complexes with kallikreins of prostatic origin.

Materials and Methods

Tissue Samples

Human tissue specimens were obtained during surgery from patients undergoing transurethral resection of the prostate because of benign enlargement ($n > 50$) or CAP ($n > 150$), or radical prostatectomy as treatment for prostate cancer ($n = 3$). For immunohistochemistry and *in situ* hybridization studies, tissues were fixed within 30 minutes of removal in Bouin's solution (for 6 hours) or 4% buffered paraformaldehyde (overnight) and embedded in paraffin. In addition, routine-fixed archival specimens were examined by a national board-certified pathologist (VG) after hematoxylin and eosin staining. Selected areas from 50 cases of BPH and 50 cases each of highly, moderately, and poorly differentiated CAP were used to construct tissue microarrays of 1.0 mm in diameter. Two different areas of well-defined morphology were chosen in each patient. All kinds of tissue samples were sectioned at 3- μ m thickness, mounted on Superfrost plus slides (Menzel-Gläser, Germany), and dried for 2 hours at 65°C. For *in situ* hybridization, each step was performed under RNase-free conditions. For Western blotting, tissue

specimens were frozen in liquid nitrogen and stored at -80°C until extraction. The Helsinki Declaration regarding the use of human tissues was strictly observed.

Cell Lines

The malignant prostatic cell lines PC-3 and DU-145 [American Type Culture Collection (ATCC), Manassas, VA] were cultured in Ham's F-12 medium and supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (Life Technologies, Paisley, UK), whereas LNCaP cells (ATCC) were maintained in RPMI 1640 medium (ATCC) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. Incubation was performed at 37°C in a humidified atmosphere of 95% air and 5% CO₂. For immunohistochemistry, cells were washed twice in PBS and centrifuged at 720 $\times g$. Cell pellets were fixed in 10% paraformaldehyde for 25 minutes and 30 to 50 μ l of Mayer's hematoxylin was added. The pellets were dehydrated in 70% ethanol (overnight). Ethanol was then added at increasing concentrations followed by xylene. Finally, cell pellets were embedded in paraffin and sectioned as described above.

Antibodies and Purified Proteins

Polyclonal rabbit IgGs raised against anti-thrombin derived from human plasma (code no. A 0296; DAKO A/S, Glostrup, Denmark), a monoclonal IgG preparation against human anti-thrombin (code no. A-5816; Sigma-Aldrich, Corp., St. Louis, MO), and monoclonal anti-PSA IgGs (code 2E9), generated as previously described,³⁵ were used. Monoclonal anti-human chromogranin A (CgA) IgGs (code no. M 869, DAKO) were used to identify neuroendocrine cells, and a monoclonal anti-CD68 IgG preparation (code no. M 814, DAKO) was used as a marker for macrophages. Fab fragments of anti-digoxigenin-AP (code no. 1093274; Roche, Germany), horseradish peroxidase-linked anti-rabbit IgG or anti-mouse IgG (code no. NA 934 or NA 931; Amersham Pharmacia Biotech, Uppsala, Sweden) were used in immunohistochemistry, *in situ* hybridization, and Western blotting studies.

Biotinylated anti-rabbit and anti-mouse IgGs, used as secondary antibodies in immunohistochemistry, were included in the DAKO ChemMate kit (code K5001; BioTek Solutions, Winooski, VT). Rabbit anti-mouse IgG (code no. Z259, DAKO), alkaline phosphatase anti-alkaline phosphatase mouse monoclonal IgG (code no. D0651, DAKO) and alkaline phosphatase-conjugated, swine anti-rabbit IgGs (code no. D 0306, DAKO) were used in the alkaline phosphatase anti-alkaline phosphatase method for immunohistochemistry. Nonimmune rabbit IgG and mouse IgG1 (code nos. X 936 and X 0931, DAKO) served as negative controls.

Anti-thrombin (native), isolated from human blood plasma³⁶ was a generous gift from Margareta Kjellberg, Department of Clinical Chemistry, University Hospital Malmö, Sweden. PSA, purified from seminal fluid, was obtained as described earlier.³⁷ Recombinant pro-PSA, as well as two different recombinant forms of mutated

pro-hK2, denoted fXahK2 (which is converted from pro-hK2 to active hK2 by factor Xa, called hK2 in this article) and ekhK2 (which may be converted from pro-hK2 to active hK2 by enterokinase, also called pro-hK2), have been described previously.^{38,39} The enzymatic activity of fXa-activated hK2 is known to be at least 80% of that of the wild-type (wt) hK2, whereas pro-hK2 (eg, ekhK2) is not enzymatically active.

Preparation of Probes for in Situ Hybridization

A 27-mer oligodeoxynucleotide probe corresponding to nucleotides 144 to 170 in the transcript for anti-thrombin, selected from known sequence data,⁴⁰ and a 30-mer oligodeoxynucleotide probe complementary to translated parts of the transcripts coding for PSA (nucleotide positions 528 to 557)⁴¹ were investigated with regard to similarities with human mammalian nucleotide sequences using the software BLAST, version 2.0.10.⁴² In addition, an oligodeoxythymidine (oligo-dT) probe (30 mer) was used as a positive control for successful tissue preparation and hybridization. All probes were purchased from DNA Technology (Aarhus, Denmark) and labeled with digoxigenin-dUTP using a 3'-end-labeling kit for tailing (kit no. 1417231, Roche), as previously described.⁴³

Immunohistochemistry

Immunohistochemistry was performed using a detection kit (DAKO ChemMate detection kit, peroxidase/diaminobenzidine tetrahydrochloride, rabbit/mouse, code no. K 5001) and a staining instrument (DAKO TechMate 500/1000, BioTek Solutions). Briefly, the sections were deparaffinized and rehydrated. For antigen retrieval, tissue sections were incubated with citrate buffer (10 mmol/L, pH 6.0) and heated in a microwave oven at 750 W for 2 × 5 minutes. The sections were incubated with primary antibodies for 60 minutes at room temperature. After washing, they were incubated with biotinylated secondary antibodies against rabbit or mouse IgG. The immunoreactivity was visualized using the manufacturer's protocol for the peroxidase/3,3'-diaminobenzidine tetrahydrochloride reagent in the ChemMate kit (BioTek), and sections were counterstained with Mayer's hematoxylin solution.

The alkaline phosphatase anti-alkaline phosphatase method was used to immunostain sections of paraffin-embedded cancer cell lines (PC-3, DU-145, and LNCaP).⁴⁴ After deparaffinization and rehydration, sections were subjected to antigen retrieval in citrate buffer (10 mmol/L, pH 6.0) and heated in a microwave oven at 750 W for 2 × 5 minutes. The sections were then thoroughly rinsed in distilled water and immersed in Tris buffer (0.05 mol/L Tris buffer, pH 7.6, 0.05% Tween) for 10 minutes. The sections were incubated overnight at room temperature with primary antibodies (DAKO) (diluted in Tris buffer with 2% bovine serum albumin), and subsequently with alkaline phosphatase-conjugated secondary antibodies (DAKO, 1:25 in Tris buffer). To visualize the immunocomplexes formed, sections were developed in

0.05 mol/L Tris (pH 8.4) buffer containing 0.13 mol/L NaCl, 0.3 mg/ml naphthol (AS-MX) phosphate (code no. N-4875) diluted in dimethylformamide, 1.5 mg/ml Fast Red, and 5 mmol/L levamisol (all from Sigma-Aldrich) for 30 minutes. The tissue sections were then washed in distilled water, counterstained with Mayer's hematoxylin solution for 1 minute and coverslip mounted in Faramount mounting medium (DAKO). All other reagents used in this process were purchased from Merck (Darmstadt, Germany).

As a negative control, adjacent sections were processed by replacing the primary antibody with non-immune rabbit IgG1 (code X 0936, DAKO) or nonimmune mouse IgG1 (code X 0931, DAKO) diluted to the same concentration as the tested primary antibody. To further evaluate the specificity of the immunostaining, purified anti-thrombin was added in 10 to 100 times molar excess to the polyclonal anti-thrombin IgG preparation for overnight incubation, and adjacent sections were subsequently immunostained with and without addition of the antigen.

For dual immunostaining, sections were first processed using polyclonal anti-AT IgGs and a DAKO ChemMate Detection Kit, and subsequently using the alkaline phosphatase anti-alkaline phosphatase method with monoclonal IgGs and Fast Blue (Sigma) for the detection of PSA, CgA, or CD68.

In Situ Hybridization

In situ hybridization with digoxigenin-labeled oligodeoxynucleotide probe was performed as previously described.⁴³ Sections were hybridized with 30 μ l of probe (200 ng/ml in hybridization buffer) overnight at 30°C and washed in 1× standard saline citrate (150 mmol/L NaCl and 15 mmol/L sodium citrate, pH 7.0) at room temperature for 10 minutes, 0.2× standard saline citrate at 43°C for 4 × 15 minutes, and 1× standard saline citrate for 10 minutes at room temperature. For the detection of hybridization signals, sections were equilibrated in buffer I (100 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl) and blocked in the same buffer with 2% normal sheep serum and 0.3% Triton X-100 for 30 minutes. After incubation with alkaline phosphatase-conjugated Fab fragments of anti-digoxigenin IgG diluted 1:500 in buffer I containing 1% normal sheep serum, 0.03% Triton X-100 for 3 hours at room temperature, sections were equilibrated in detection buffer (100 mmol/L Tris-HCl, pH 9.5, 100 mmol/L NaCl, 50 mmol/L MgCl₂) and developed in detection buffer containing 0.33 mg/ml nitro blue tetrazolium chloride, 0.175 mg/ml 5-bromo-4-chloro-3-indolyl phosphate, and 1 mmol/L levamisol. The staining reaction was stopped in ethylenediaminetetraacetic acid buffer (10 mmol/L Tris-HCl, pH 7.5, 0.9% NaCl, 10 mmol/L ethylenediaminetetraacetic acid) after 15 to 20 hours. The slides were coverslipped with Faramount mounting medium (DAKO). All other reagents were purchased from Sigma-Aldrich, Merck, or Amersham Pharmacia Biotech. For control purposes, adjacent sections were hybridized with digoxigenin-labeled oligo-dT probe to assure successful fixation, and hybridization buffer was used as a negative control.

Protein Extraction of Tissue Specimens

Benign or malignant prostatic tissue was homogenized at 0°C in buffer containing 0.5 mol/L NaCl, 25 mmol/L benzamidine hydrochloride, and 50 mmol/L 2-(*N*-morpholino) ethane sulfonic acid. Homogenates were centrifuged at $34,540 \times g$ for 15 minutes at 4°C and the supernatants were subsequently centrifuged at $154,000 \times g$ for 1 hour, using an ultracentrifuge (Beckman SW41TI) at 4°C. Pellets were extracted in the buffer described above supplemented with 0.5% Triton X-100, and centrifuged at $34,540 \times g$ for 15 minutes at 4°C. Protein concentrations were measured using a bicinchoninic acid protein assay kit with bovine serum albumin as standard, according to the manufacturer's instructions (kit no. 23225; Pierce, Rockford, IL).

Preparation of Complexes in Vitro

Purified anti-thrombin and hK2 or PSA were incubated at 4:1 or 11:1 molar ratio, respectively, in 0.05 mol/L of Tris-HCl buffer (pH 7.4) containing 0.5 mol/L NaCl, for 30 minutes, 3 hours, or 30 hours at 37°C, with and without 4 IE/ml heparin (Amersham Pharmacia Biotech). PSA and hK2 were replaced by enzymatically inactive pro-PSA and pro-hK2, respectively, in negative control experiments.

Kinetic Analysis of PSA or hK2 Inhibition by Anti-Thrombin

Anti-thrombin and PSA were incubated for 30 minutes (11:1 molar ratio), whereas anti-thrombin and hK2 were incubated for 1, 5, or 30 minutes with and without 4 IE/ml heparin at 37°C, in 0.05 mol/L Tris-HCl buffer (pH 7.4) containing 0.5 mol/L NaCl. The inhibition of 500 ng of PSA or 200 ng of hK2 by anti-thrombin was monitored using 200 μ mol/L of PSA substrate Mu-Ser-Ser-Tyr-Tyr-AMC (lot no. 14245; Livermore Laboratory, CA) or 100 μ mol/L of hK2 substrate H-Pro-Phe-Arg-AMC (lot no. 511961; Bachem AG, Bubendorf, Switzerland) in 200 μ l of 50 mmol/L Tris buffer (pH 7.5) containing 0.1 mol/L NaCl and 0.2% bovine serum albumin. Substrate hydrolysis was monitored for 10 minutes in microtiter plates. The inhibition was compared with that in enzyme-free control experiments.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blotting

Benign and malignant prostate tissue homogenates, as well as anti-thrombin/PSA and anti-thrombin/hK2 incubation mixtures, were subjected to 12% SDS-PAGE under reduced conditions⁴⁵ and run with 25 mmol/L of Tris buffer containing 192 mmol/L glycine and 0.1% SDS. After electrophoresis, the proteins were transferred onto Hybond ECL nitrocellulose membranes (Amersham Pharmacia Biotech) in 25 mmol/L of Tris buffer with 192

mmol/L of glycine and 20% methanol, according to the manufacturer's instructions. Nonspecific binding of proteins was blocked with 5% nonfat milk powder in PBS containing 0.1% Tween 20, for 1 hour at room temperature. The membranes were washed three times in PBS containing 0.05% Tween 20 (PBS-T). The membranes were subsequently incubated with polyclonal or monoclonal anti-thrombin IgGs for 1 hour. After washing in PBS-T, the membranes were incubated with horseradish peroxidase-linked anti-rabbit or anti-mouse IgGs diluted 1:10,000 in blocking solution. After washing in PBS-T, the proteins were visualized with enhanced chemiluminescent (ECL+) Western blotting detection reagents (code no. RPN 2132, Amersham Pharmacia Biotech). To stain the proteins, gels were stained with Coomassie Brilliant Blue R-250 (Bio-Rad, Hercules, CA) after electrophoresis. Protein standards with known molecular weights (code no. 161-0372, Bio-Rad) were used to estimate the protein size.

Results

Immunohistochemistry

Anti-thrombin immunoreactivity was demonstrated in the benign and malignant prostate gland, as well as in all three malignant prostatic cell lines (Figure 1). In the benign epithelium, basal cells were frequently immunostained and this became obvious in glandular ducts where basal cell proliferation is sometimes observed. Luminal cells were less frequently found to be anti-thrombin-positive in peripheral parts of the prostatic glandular tree and in BPH nodules, but prostatic ducts with specific anti-thrombin immunostaining in luminal cells were also observed. PSA immunoreactivity was confined to the luminal cells as expected. In dual-staining experiments, a subpopulation of luminal cells was immunostained for both anti-thrombin and PSA, but most luminal cells were only positive for PSA (data not shown). Scattered anti-thrombin immunoreactive cells were demonstrated in the prostatic stroma, a majority of which was identified as macrophages, because they were CD-68 immunoreactive. Anti-thrombin immunostaining was not observed in any case in CgA-positive neuroendocrine cells in dual-immunostaining experiments.

The anti-thrombin immunoreactivity of the malignant prostate gland was, in general, more pronounced with regard to both signal intensity and frequency of positive cells compared to the BPH specimens (Figure 1). Dual immunostaining revealed a minor subpopulation of tumor cells, positive to both anti-thrombin and PSA, whereas the majority of tumor cells was found to be positive to either anti-thrombin or PSA (Figure 1).

Androgen-independent prostatic tumor cells (PC-3 and DU-145) and the androgen-dependent cell line (LNCaP) contained immunoreactive anti-thrombin, whereas monoclonal anti-PSA IgGs showed positive signals only in LNCaP cells (Figure 1).

The tissue microarray from 200 patients yielded 158 evaluated spots in immunohistochemistry (Figure 2 and Table).

Figure 1. Immunohistochemical localization of anti-thrombin in paraffin sections of prostatic tissues and malignant prostatic cell lines. Polyclonal anti-thrombin, monoclonal anti-CgA, anti-CD68, and anti-PSA IgGs were used. **A:** Strong immunoreaction to anti-thrombin in prostate cancer. **B:** Negative control on adjacent paraffin section when excess purified anti-thrombin was added to polyclonal anti-thrombin IgGs before immunostaining. **C:** Benign prostate gland. Anti-thrombin immunostaining common in basal epithelial cells (**single arrows**), luminal cells (**double arrows**), and in scattered cells in the stroma(s). **D:** Dual immunostaining for anti-thrombin (brown) and PSA (blue) in prostate cancer. A population of tumor cells was positive to both anti-thrombin and PSA (**arrow**), whereas other tumor cells contained either anti-thrombin or PSA. **E:** Benign prostatic tissue section. Stroma cells were immunostained for either anti-thrombin (brown, **single arrow**) or CD-68 (macrophages) (blue, **single arrow**), or both AT and CD-68 (**double arrow**). **F:** Benign prostatic epithelium with dual immunostaining for anti-thrombin (brown) and CgA (neuroendocrine cells) (blue). No co-localization was observed. LNCaP cells (**G**) and PC-3 cells (**I**) are intensely immunostained for anti-thrombin. A subpopulation of LNCaP cells is PSA immunoreactive (**H**), but PC-3 cells are not (**J**). The immunoperoxidase method with 3,3'-diaminobenzidine tetrahydrochloride (brown) and the alkaline phosphatase anti-alkaline phosphatase method with Fast Blue or Fast Red as chromophores were used. Scale bars: 40 μ m (**A, B, D, E, G-J**); 80 μ m (**C**); 20 μ m (**F**).

Figure 2. Paraffin sections of tissue microarrays. **A:** Low-magnification micrograph showing intense immunostaining for anti-thrombin in tumor cells (**small arrows**) compared to glands with PIN lesions (**large arrows**). **B:** Higher magnification in a different tissue section demonstrating prostatic tumor cells with various staining intensities for anti-thrombin. **Arrow** indicates strong immunostaining for anti-thrombin in a tumor cell, characterized by vacuolization (**arrow**). **C:** Different immunostaining pattern for PSA in a tissue section adjacent to **B**. Immunoperoxidase with 3,3'-diaminobenzidine tetrahydrochloride. Scale bars: 200 μ m (**A**); 80 μ m (**B**, **C**).

All tissue sections of BPH, prostatic intraepithelial neoplasia (PIN), and CAP were positive with regard to anti-thrombin, whereas one tumor was negative for PSA. Again, the anti-thrombin immunostaining intensity in tumor cells was usually stronger than in BPH and PIN areas, however, non-stained tumor cells were also observed. The immunostaining intensity increased gradually from tumors of Gleason grade 1 to 3. In tumor areas classified as Gleason grades 4 and 5, cells capable of forming vacuoles were intensely stained, whereas several other tumor cells were weakly stained or devoid of immunostaining.

Monoclonal and polyclonal IgG preparations against anti-thrombin generated identical staining patterns, but the signal-to-noise ratio was better using polyclonal IgGs. No immunostaining was detected when adjacent tissue sections were incubated with nonimmune IgGs or when excess antigen was added to the primary IgG preparation (Figure 1).

In Situ Hybridization

Digoxigenin-labeled oligodeoxynucleotide probes specific to anti-thrombin or PSA were added to adjacent paraffin sections generating hybridization signals with a distribution pattern in accordance with the immunohistochemical findings in the benign and malignant prostatic epithelium (Figure 3). In BPH areas, hybridization signals for anti-thrombin mRNA were stronger in basal cells and in scattered stromal cells than in luminal cells. Hybridization signals were stronger in malignant epithelial cells

than in benign cells. In adjacent sections hybridized with the PSA-specific probe, positive signals were restricted to luminal cells in the epithelium (Figure 3).

SDS-PAGE and Western Blotting

Benign and malignant prostate tissue homogenates were subjected to SDS-PAGE and immunodetected with anti-thrombin-specific IgG preparations. A major band at 58 kd, corresponding in size to purified anti-thrombin, was detected visually using polyclonal and monoclonal anti-thrombin IgGs (Figure 4). Two additional components, between 50 kd and 75 kd in size, were also detected by polyclonal but not by the monoclonal anti-thrombin-specific IgGs.

Analysis of Complex Formation with PSA and hK2

Anti-thrombin/PSA and anti-thrombin/hK2 incubation mixtures were analyzed with 12% SDS-PAGE under reduced conditions and subsequently stained with Coomassie Brilliant Blue R-250 (Figure 5a) or detected using Western blotting (Figure 5b). Anti-thrombin/hK2 complexes (between 75 kd and 100 kd) were readily formed after 30 minutes of incubation at 37°C. However, anti-thrombin formed complexes less readily with PSA; PSA-anti-thrombin complexes (between 75 kd and 100 kd) only being detect-

Table 1. Summary of Tissue Microarray Data Regarding Anti-Thrombin and PSA in BPH, PIN, and CAP (Gleason Grade) Obtained by Immunohistochemistry

Diagnosis	Number of cases evaluated	AT		PSA	
		Positive	Negative	Positive	Negative
BPH	46	46	0	46	0
PIN	17	17	0	17	0
CAP					
Gleason grade 2	27	27	0	27	0
Gleason grade 3	40	40	0	40	0
Gleason grade 4	32	32	0	32	0
Gleason grade 5	13	13	0	12	1

Figure 3. Localization of anti-thrombin and PSA transcripts in prostatic tissues by nonradioactive *in situ* hybridization. **A:** Paraffin section from benign prostatic tissue hybridized with a digoxigenin-labeled anti-thrombin oligodeoxynucleotide probe showing stronger signals in basal epithelial cells and a few stroma cells (**arrow**), than in luminal cells. **C:** Increased expression of anti-thrombin in malignant (m) *versus* benign (b) epithelium. **E:** Strong hybridization signals in luminal but no detectable signal in basal cells (**arrow**) of the benign prostatic epithelium using a PSA-specific oligodeoxynucleotide probe. **B** and **D** represent negative controls where the anti-thrombin-specific oligodeoxynucleotide probe was replaced by buffer in adjacent tissue sections. Alkaline phosphatase-conjugated Fab fragments of anti-digoxigenin IgGs were used for detection with 5-bromo-4-chloro-3-indolyl phosphate-nitro blue tetrazolium chloride as substrates. Scale bars: 40 μ m (**A**, **B**); 200 μ m (**C**, **D**); 20 μ m (**E**).

able after 30 hours of incubation at 37°C. Both complexes (between 75 kd and 100 kd) could be recognized by polyclonal as well as by monoclonal IgGs against anti-thrombin. In an anti-thrombin/hK2 incubation mixture additional components between 50 kd and 75 kd in size (apart from the anti-thrombin band), were also recognized by the polyclonal anti-thrombin-specific IgGs. One of them was also identified by the monoclonal anti-thrombin-specific IgGs. The addition of 4 IE/ml heparin increased the amount of anti-thrombin/hK2 complexes formed but did not promote the formation of complexes between anti-thrombin and PSA. There were no detectable complexes in anti-thrombin/pro-PSA and anti-thrombin/pro-hK2 incubation mixtures.

Kinetic Analysis of PSA and hK2 Inhibition by Anti-Thrombin

The enzymatic activity of hK2 after incubation with anti-thrombin for 1, 5, and 30 minutes at a molar ratio of 11:1 (anti-thrombin:hK2) was measured in microtiter plates using an hK2 substrate assay (Figure 6). Anti-thrombin completely inhibited hK2 activity at (11:1) molar excess, and heparin accelerated the rate at which anti-thrombin

inhibited hK2 activity. Similarly, the inhibitory action of anti-thrombin on incubation with PSA (11:1 molar ratio) was studied with a PSA substrate assay, showing that anti-thrombin did not significantly inhibit PSA activity after 30 minutes, or when 4 IE/ml heparin was added (Figure 7). However, after an extended time period, 30 hours, this molar excess of anti-thrombin was capable of completely inhibiting the enzymatic activity of PSA (data not shown).

Discussion

As in other malignancies, angiogenesis has been suggested to play an important role in tumor growth in CAP. For instance, plasma levels of vascular endothelial growth factor are higher in patients with metastatic CAP⁴⁶ and the expression of vascular endothelial growth factor receptors has been demonstrated in prostatic tumors.^{47,48} Anti-thrombin, a well-characterized member of the serpin superfamily, was recently reported to possess anti-angiogenic and anti-tumor properties.^{9,10,15} It has been reported that anti-thrombin occurs in tumors such as hepatocellular carcinoma,¹² in the stromal compart-

Figure 4. Western blotting of benign and malignant prostatic tissue homogenates (3 μ g) with anti-thrombin IgGs under reduced conditions. **Lane 1**, Purified anti-thrombin (positive control); **lane 2**, benign tissue; **lane 3**, malignant tissue, all lanes incubated with polyclonal anti-thrombin IgG; **lane 4**, malignant tissue; **lane 5**, benign tissue, incubated with monoclonal anti-thrombin IgGs. Immunocomplexes were detected with the ECL system. The positions of standard proteins of known molecular weights (kd) are indicated.

ment of small-cell lung cancer¹³ and recently anti-angiogenic anti-thrombin was also derived from a pancreatic tumor inoculum in an *in vivo* animal model.¹⁵

The aim of our study was to determine whether anti-thrombin is expressed in prostatic tissues and, if so, whether anti-thrombin plays a role in the benign and malignant prostate, as proposed for other malignancies.

The molecular mechanisms underlying the oncogenesis and progression of prostate cancer are still poorly understood.^{49,50} The detection of prostate cancer relies heavily on measurements of PSA in serum.^{21,22} PSA exists in different molecular forms and measurements of free and complexed forms of PSA in blood are widely used to enhance the specificity of prostate cancer detection.^{26,27} More recently, attention has been paid to hK2 as a new potential marker to increase the specificity of the diagnosis of BPH and CAP.^{29–31} Both PSA and hK2 are members of the glandular kallikrein family and are known to form complexes with different proteinase inhibitors.^{26–33} The degree of complex formation between PSA and α -1-anti-chymotrypsin in blood is higher in CAP than in BPH.^{26–28} hK2 has been shown to form complexes with α -2-anti-plasmin, plasminogen activator inhibitor-1, and protein C inhibitor,^{30–32} which may be of importance in tumor invasion.

In the present study, the major findings were that anti-thrombin is expressed in benign and malignant prostatic tissue and prostate cancer cell lines (PC-3, DU-145, and LNCaP) and that complex formation readily occurs with hK2, but much less readily with PSA. Different techniques were used for the detection of anti-thrombin expression. Immunohistochemistry and *in situ* hybridization studies revealed the presence of anti-thrombin in benign and malignant prostatic tissues. In benign prostatic epithelium, anti-thrombin is widely distributed in basal cells and less frequently found in luminal cells (Figures 1 and 3), but in some specimens, glandular ducts with virtually all luminal cells immunostained were observed. In PIN lesions, anti-thrombin staining was preserved in the basal

Figure 5. a: SDS-PAGE under reduced conditions of anti-thrombin incubated with PSA or hK2 at 37°C without (**lanes 1 to 4**) and with (**lanes 5 to 8**) heparin (4 IE/ml). **A: Lane 1**, anti-thrombin incubated with PSA for 30 hours, the upper band represents anti-thrombin/PSA complex; **lane 2**, anti-thrombin/PSA for 30 minutes; **lane 3**, anti-thrombin/pro-PSA for 30 hours, no complex formed; **lane 4**, purified anti-thrombin; **lanes 5 to 8**, anti-thrombin incubated in the same manner as in **lanes 1 to 4**, but in the presence of 4 IE/ml heparin. **B: Lane 1**, anti-thrombin/hK2 incubated for 3 hours, the upper band represents anti-thrombin/hK2 complex; **lane 2**, anti-thrombin/hK2 for 30 minutes, shows detectable complex; **lane 3**, purified anti-thrombin; **lane 4**, anti-thrombin/pro-hK2 for 3 hours, no complex formed; **lanes 5 to 8**, anti-thrombin incubated in the same manner as in **lanes 1 to 4**, but with 4 IE/ml heparin. Identical standard proteins with known molecular weights (kd) were used in **A** and **B** (**lanes S**). Coomassie Brilliant Blue R-250 staining was used. **b:** Detection of anti-thrombin/PSA and anti-thrombin/hK2 complexes by Western blotting under reduced conditions. **Lane 1**, purified anti-thrombin; **lane 2**, anti-thrombin/hK2 (30 minutes of incubation); and **lane 3**, anti-thrombin/PSA (30 hours of incubation). **Lanes 4** and **5** as **lanes 2** and **3**, respectively. Immunocomplexes were formed with polyclonal IgGs against anti-thrombin in **lanes 2** and **3** and with monoclonal IgGs against anti-thrombin in **lanes 4** and **5**. The ECL system was used for detection. The positions of standard proteins (kd) are indicated.

cell compartment, and anti-thrombin staining in the luminal cells was comparable to that in BPH areas (Figure 2). Analyses of tissue microarrays showed that all BPH, PIN, and CAP tissue spots contained anti-thrombin-immunoreactive cells, and more intense staining was observed in populations of tumor cells than in BPH and PIN areas (Table 1 and Figure 2). The expression of anti-thrombin was particularly strong in Gleason grade 3 tumors, but was also preserved in cells characterized by vacuolization in poorly differentiated tumors. Dual-immunostaining experiments revealed tumor cells expressing PSA but devoid of anti-thrombin immunostaining, and vice versa.

As described in previous reports, PSA was restricted to the luminal cells of prostatic epithelium and was not found in the basal cells or in the prostatic stroma compartment. Dual-immunostaining experiments also clearly demonstrated different immunolocalization patterns for anti-thrombin and PSA in benign tissues (data not shown). The immunostaining pattern of anti-thrombin is similar to those we have described earlier for α -1-anti-chymotrypsin and trypsinogen.^{44,51,52} Furthermore, anti-thrombin was not found in CgA-immunoreactive neuroendocrine cells, and anti-thrombin-containing stroma cells

Figure 6. Inhibition of hK2 by anti-thrombin. **A:** The cleavage of 100 $\mu\text{mol/L}$ of H-Pro-Phe-Arg-AMC by hK2 incubated with anti-thrombin at 37°C with and without 4 IE/ml heparin for 1 minute or 5 minutes. **B:** Same experiment after incubation for 30 minutes. The measurement interval was 1 minute in microtiter plates in all cases.

were, in part, identified as CD-68-positive macrophages (Figure 1).

Because PSA immunoreactivity is particularly strong in the luminal cells of BPH epithelium, different mechanisms seem to regulate anti-thrombin and PSA expression. This is also strongly indicated by the present reported findings that anti-thrombin is expressed by both androgen-independent cell lines (PC-3, DU-145) that do not express PSA, and an androgen-dependent cell line (LNCaP), that expresses PSA (Figure 1).

Results from nonradioactive *in situ* hybridization studies demonstrated a staining pattern in accordance with the immunocytochemical findings in benign as well as in malignant tissue specimens (Figure 3). The intense immunostaining and *in situ* hybridization signals for anti-thrombin in some tumor cells suggest up-regulation, whereas expression seems to be down-regulated in the luminal cells of BPH epithelium. This was further supported by strong immunostaining in all malignant cell lines examined (Figure 1). In contrast, overexpression of PSA in malignant *versus* benign tissue was not observed, again indicating that tissue expression of anti-thrombin and PSA is regulated by different mechanisms.

Western blotting with polyclonal and monoclonal anti-thrombin IgGs further supported the histochemical findings. In tissue homogenates from CAP and BPH, the predominant band was detected at 58 kd, corresponding in size to purified anti-thrombin. This band seems to

Figure 7. Inhibition of PSA by anti-thrombin. The cleavage of 200 $\mu\text{mol/L}$ of Mu-Ser-Ser-Tyr-Tyr-AMC by PSA incubated with anti-thrombin for 30 minutes at 37°C with and without 4 IE/ml heparin. The measurement interval was 1 minute.

contain an additional immunoreactive component of somewhat lower molecular weight and may represent cleaved anti-thrombin, but further investigations are needed to clarify the exact nature of this component.

PSA has been widely used as a diagnostic marker of prostate cancer and hK2 has recently been suggested as a potential marker for this disease.^{21–23,29–31} Here we were interested in the possible interaction between anti-thrombin and PSA or hK2. Both kinetic analysis and protein staining (Coomassie Brilliant Blue R-250) indicated that anti-thrombin rapidly inhibited hK2 *in vitro* and such inhibition was accelerated by heparin, whereas anti-thrombin did not inhibit PSA after 30 minutes, even when heparin was added (Figures 5 to 7). The formation of an anti-thrombin/PSA complex required a longer incubation time and optimized conditions. Both complexes were recognized by polyclonal and monoclonal IgGs against anti-thrombin (Figure 5). Through the formation of complexes, anti-thrombin expressed in the human prostate gland may act as a local inhibitor of hK2 *in vivo*. A functional role of anti-thrombin in regulating the proteolytic activity of hK2 may be of importance under benign as well as under malignant conditions. Apart from the fact that anti-angiogenic and anti-tumor growth properties have been attributed to anti-thrombin, it may also act as a local inhibitor of tumor invasion because of its ability to form complexes with proteases, including plasma kallikreins and trypsin.^{4,6}

Because anti-thrombin has been shown to act as a local anti-angiogenic and anti-tumor factor in other tumors, further investigation of highly expressed anti-thrombin in CAP may improve the prospects of finding new treatment options in prostate cancer. The fact that blood-clotting factors may participate in the regulation of glandular kallikreins and in angiogenesis indicates that certain anti-proteases may participate in different cascade reactions.

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