Enhancement of the Expression of Urokinase-Type Plasminogen Activator from PC-3 Human Prostate Cancer Cells by Thrombin

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ABSTRACT

The presence of procoagulants and fibrin deposition have been demonstrated in malignant tumors. Although thrombin, a key enzyme in coagulation, has other various biological functions, the significance of its presence in tumors is not known. We studied the effects of thrombin on the expression of urokinase-type plasminogen activator (uPA) which is known to play a role in tumor invasion, using a human prostate cancer cell line PC-3. Human α-thrombin added to cultures of PC-3 produced a dose-dependent and time-dependent increased secretion of uPA that was greatest at 3–6 h after exposure to thrombin. Increase in uPA antigen paralleled the increase in mRNA level, which reached a maximum at 4 h. Thrombin showed the maximum effect on uPA expression at a concentration 1–2 units/ml. Zymography showed that transient exposure to thrombin induced an increase in fibrinolytic activity which could be quenched by anti-uPA antibody. The thrombin receptor-activating peptide also caused an increase in uPA protein and mRNA level, indicating the presence of the same thrombin specific receptor on PC-3 cells as on platelets and endothelial cells. Thrombin did not affect the expression of other components of the plasminogen activation system, tissue-type plasminogen activator and type-1 plasminogen activator inhibitor, and uPA receptor. These results indicate that thrombin increases uPA expression selectively by the stimulation of a functional thrombin receptor on PC-3 cells. Since uPA is known to play a role in pericellular proteolysis of extracellular matrix, thrombin may be involved in the regulation of tumor invasion and metastasis.

INTRODUCTION

The presence of the coagulation process in malignant tumors has been well recognized. Fibrin deposition has been demonstrated in the stroma around the invading front of the tumor (1, 2) and on circulating cancer cells in blood (3). The presence of procoagulants has also been demonstrated in cancer tissues (4). In the process of coagulation, thrombin is generated which, in addition to fibrin formation, has other various biological functions such as an increase in vascular permeability, stimulation of adherence of platelets and endothelial cells, chemotaxation of monocytes, and mitogenic activity on endothelial cells and fibroblasts (5). Thus, it is likely that thrombin generated in the vicinity of cancer cells may affect tumor cell behavior. However, the significance of thrombin or other components of the coagulation system is not fully understood.

On the other hand, the plasminogen-plasmin system has been shown to play a role in tumor growth, invasion, and metastasis (6). uPA is believed to be an initiator of pericellular proteolysis of the extracellular matrix. This is supported by the fact that anti-uPA could reduce tumor invasion and metastatic ability (7). uPA has been also found to have a mitogenic effect on several cell lines (8, 9).

In view of these findings, it is possible that thrombin may affect tumor cell behavior by modulating the expression of the components of the plasminogen-plasmin system. In the present study, we focused on the effect of thrombin on uPA expression in cancer cells and examined three prostate cancer cell lines: PC-3, a high uPA producer; DU-145, which is a low uPA producer; and LNCaP, a non-uPA producer. We demonstrate here that thrombin induced uPA production by PC-3 cells through the stimulation of a thrombin-specific receptor.

MATERIALS AND METHODS

Materials. Human α-thrombin (3250 NIH units/mg) was purchased from Sigma Chemical Co. (St. Louis, MO). Cycloheximide and actinomycin D were purchased from Sigma Chemical Co., and heparin was purchased from Elkins-Sinn, Inc. (Cherry Hill, NJ). Native hirudin was purchased from American Diagnostica, Inc. (St. Louis, MO), and [32P]dCTP was purchased from Amersham (Arlington Heights, IL). Antithrombin III was kindly provided by Dr. T. Yin, Haemachem, Inc. (St. Louis, MO). RPMI-1640 and fetal bovine serum were obtained from Central facility, Northwestern University Cancer Center. PPACK was purchased from Chemica Alta, Ltd. (Edmonton, Alberta, Canada).

PPACK-thrombin was prepared by dissolving 50 μg of PPACK and 3.3 μg of thrombin in 5 ml of 0.3 M sodium phosphate buffer (pH 7.4) and dialyzing against 0.1 M sodium phosphate buffer-0.15 M NaCl (pH 7.4) to eliminate free PPACK. The activity of PPACK-thrombin was less than 1% of that of original thrombin by clotting time.

The human uPA probe was prepared by PCR from pRSV-uPA (10), which is a gift from Dr. F. Blasi (University of Milano, Italy). uPA gene fragment (from +5433 to +6148 in exon XI) was amplified by PCR with a set of oligonucleotide primers, and then the products were used as a template for second PCR. The final product showed a single 0.7-kilobase band in agarose gel and was used as uPA probe. Human GAPDH complementary DNA probe was a gift from Dr. G. Soff (Northwestern University). TRAP (SFLRLRPNFDKYEPFWFE) and a CP (SFLRLRPNFDKYEPFWFE) in which the first two amino acids serine and phenylalanine, are transposed were synthesized, based on the report by T. K. Vu et al. (11) at the Biotechnology Facility in Northwestern University, Chicago, IL.

Cell Culture. The PC-3 cell line is a prostate cancer cell line derived from a lumbar vertebral metastasis of a poorly differentiated human prostate adenocarcinoma. These cells are androgen independent, readily metastasize when inoculated into nude mice, and are biologically aggressive (12). Cells were grown to confluence on 24-well plates (Corning, Corning, NY) in RPMI-1640 with 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml). Studies were performed by washing confluent cultures twice with RPMI-1640 and incubating the cultures at 37°C in 1 ml RPMI-1640 containing the indicated concentration of human α-thrombin and 0.1% bovine serum albumin to exclude non-specific protein effect. After incubation, the conditioned medium was collected, centrifuged at 8000 × g to remove cell debris, and stored at −70°C until assayed. The cell number was counted using a hemocytometer.

Measurement of uPA Antigen. The level of uPA antigen in the conditioned medium was measured by an ELISA (TintElize uPA kit, Biopool; Meditech, Ventura, CA) according to the manufacturer's description. TPA and

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4. The abbreviations used are: uPA, urokinase-type plasminogen activator; tPA, tissue-type plasminogen activator; scuPA, single-chain uPA; tcuPA, two-chain uPA; PPACK, phenylalanyl-prolyl-arginyl-chloromethyl ketone; PCR, polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TRAP, thrombin receptor activating peptide; CP, control peptide; EUSA, enzyme-linked immunosorbent assay; PAI-1, type-1 plasminogen activator inhibitor; SDS, sodium dodecyl sulfate.

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with 12% acrylamide gels according to Laemmli (13). Human high molecular weight uPA (American Diagnostics, Greenwich, Cr) and single chain tPA represents the mean of quadruplicate determinations from a single experiment. The result represents three separate experiments. The average secretion rate calculated from the above results is

<table>
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<th>Av. rate of uPA secretion (ng/h/10^5 cells)</th>
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<tr>
<td>0-3 h</td>
</tr>
<tr>
<td>Thrombin</td>
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<td>None</td>
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PAI-1 antigen levels were also measured by TintElize kits. TintElize PAI-1 kit detects PA/PAI-1 complexes with the same efficiency as free PAI-1.

Zymography. SDS-polyacrylamide gel electrophoresis was performed with 12% acrylamide gels according to Laemmli (13). Human high molecular weight uPA (American Diagnostics, Greenwhich, CT) and single chain tPA (American Diagnostica) were run in parallel for a standard marker. The fibrin agar indicator gels contain 1% agarose, 20 µg/ml plasmogen, 0.5 NIH units/ml thrombin, and 4 mg/ml fibrinogen. After electrophoresis, the SDS gels were soaked in 2.5% Triton X-100 for 1 h to remove the SDS and applied to the surface of the fibrin agar indicator gel. The gel was allowed to develop at 37°C.

RNA Isolation and Northern Blot Analysis. Total RNA was isolated from PC-3 cells by treatment with guanidinium isothiocyanate-sarcosyl solution followed by phenol extraction and ethanol precipitation. For Northern analysis, 12 µg RNA per lane was electrophoresed through 1.2% formaldehyde agarose gels, transferred to nylon membrane (Boehringer Mannheim, Mannheim, Germany), and cross-linked with UV light. The blots were prehybridized in 30% formamide, 5 X Denhardt's solution, 0.1% SDS, and 100 µg/ml of salmon sperm DNA for 4 h at 42°C. The prehybridization mixture was replaced with fresh solution containing labeled uPA probe prepared by nick translation using [32P]dCTP. Hybridization was performed overnight at 42°C and followed by a wash. The filters were exposed to Fuji Imaging plate BAS III for 3 h and analyzed by Fujix Bio-Imaging Analyzer BAS 2000 (Fuji, Kanagawa, Japan). After autoradiography, the filter was treated with 50% formamide-1 mM EDTA-0.1 mM Tris for 30 min at 85°C to remove uPA probe and then rehybridized with GAPDH probe under the same experimental conditions.

RESULTS

In order to examine the cell-specific expression and its responsiveness to thrombin, we determined the uPA protein levels in the medium conditioned by three prostate cancer cell lines, PC-3, DU-145, and LNCaP cells, after incubation with various concentrations of thrombin for 16 h in serum-free conditions. Nonstimulated control PC-3 cells produce about 8 ng uPA/10^5 cells/16h, while thrombin stimulation enhanced more than a 100% increase in uPA secretion. Nonstimulated DU-145 cells produce only 0.05 ng/10^5 cells/16h, and this production was not affected by thrombin stimulation. LNCaP did not secrete detectable levels of uPA with or without thrombin.

Northern blot analysis also showed that thrombin had no effect on uPA mRNA level in DU-145 and LNCaP. Therefore, we chose PC-3 cells for further study of the detailed mechanism involved in thrombin regulation of uPA.

Effect of Thrombin on uPA Secretion. Fig. 1 shows the time-course of thrombin stimulation of uPA antigen level in medium conditioned by PC-3 cells. Preliminary experiments of the dose-dependent effect of thrombin on uPA production for 24 h showed a maximal effect at a concentration of 1–2 units/ml. Thus, we used 2 units/ml thrombin for the time-course study. PC-3 cells produce increasing amounts of uPA in the medium over the course of 24 h of the experiment. The addition of thrombin resulted in more than 100% increase in the secretion of uPA antigen. During the first 3 h, there was no difference between the thrombin-stimulated cells and the control. Over the next 3 h between 3 and 6 h, the average rate of secretion, as indicated by the slope, increased to 2.43 ng/10^5 cells, which is 3-fold greater than control, and then it declined to 0.97 ng/10^5 cells between 12 and 24 h, as shown in the legend. This 3-h time lag suggests that thrombin induced uPA protein synthesis and not the release of stored uPA. The other components of the plasminogen-plasmin system, i.e., tPA, PAI-1, and PAI-2, were not detectable by ELISA.

Thrombin has been known to stimulate cell proliferation in several cell types (5). To select an optimum incubation time for further study, we studied the growth effect of thrombin on PC-3 cells. No significant increase in cell numbers were found, nor were there any morphological changes between thrombin-stimulated cells and nonstimulated cells observed within 24 h. Prolonged serum-free conditions reduce the viability of PC-3 cells. Samples were collected at 6 h after thrombin stimulation, when the maximum increase rate was observed (Fig. 1). Since the cell numbers remained constant during this period in both the thrombin-treated and control groups, we have chosen to use the end point as uPA antigen/ml in the following experiments.

Fig. 2 shows the concentration dependence of the thrombin stimulation of uPA secretion. The effect of thrombin on uPA secretion was
faint lytic zones were observed. These two zones were confirmed to be tPA and probably tPA/PAI complex since these two lytic zones disappeared when anti-tPA antibody (final concentration, 10 μg/ml) was incorporated in the indicator gel. This finding indicates that the continuous presence of thrombin is not necessary to stimulate uPA production, suggesting that thrombin stimulation triggers directly or indirectly intracellular signal transduction followed by RNA synthesis, protein synthesis, and secretion.

As shown in Table 1, when cultures were treated with cycloheximide, a protein synthesis inhibitor, the effect of thrombin on uPA secretion was completely blocked. Treatment with actinomycin D, an RNA synthesis inhibitor, also blocked the thrombin effect completely. These findings and the 3-h time lag before the increase in uPA secretion (Fig. 1) indicate that the stimulation of uPA release by thrombin requires concomitant RNA and protein synthesis.

Effect of Thrombin on uPA mRNA Level. To confirm the above result, we carried out Northern blot analysis. Fig. 4 shows the effect of various concentrations of thrombin on uPA mRNA expression. Increased mRNA level was detectable at 0.01 unit/ml thrombin, and 1 unit/ml thrombin produced the strongest signal for uPA. Thrombin (10 units/ml) resulted in a lesser message level. This down-regulation of mRNA level is in agreement with the uPA protein level as shown in Fig. 2. Fig. 5 shows a transient increase in uPA mRNA levels in response to thrombin stimulation. The message level was increased during the first 4 h and then declined. The maximal mRNA level at 4 h was in good agreement with an increased rate of protein secretion to the medium between 3 and 6 h, as shown in Fig. 1. Moreover, we examined the mRNA level of uPA receptor and PAI-1 before and after thrombin stimulation. Thrombin did not affect the mRNA levels of uPA receptor or PAI-1 in PC-3 cells. To test whether protein synthesis

<table>
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<th>Table 1 Effect of cycloheximide and actinomycin D on the thrombin induction of uPA from PC-3 cells</th>
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<td>Confluent cells were incubated for 1 h with 25 μM cycloheximide, 0.2 μM actinomycin D, or no inhibitor, and then for an additional 6 h with 2 units/ml thrombin. uPA antigen in the medium was measured by ELISA and is presented as the mean ± SE of quadruplicate determinations from a single experiment and are representative of three separate experiments.</td>
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<td>Sample</td>
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<td>None</td>
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<td>Thrombin (2 units/ml)</td>
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<td>+ Cycloheximide (25 μM)</td>
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<td>+ Actinomycin D (0.2 μM)</td>
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is required for a thrombin effect on the uPA mRNA level, PC-3 cells were incubated for 4 h with cycloheximide in combination with thrombin. A similar increase in the uPA mRNA level as when incubated with thrombin alone was observed, indicating that de novo protein synthesis is not required.

**Effect of Thrombin Inhibitors on uPA Secretion.** To determine whether enhanced expression of uPA by thrombin was actively site dependent, several kinds of thrombin inhibitors were used: (a) antithrombin III and heparin, which are physiological inhibitors; (b) hirudin, which inhibits both active site and receptor binding site of thrombin; and (c) PPACK, which blocks the active site but not binding to thrombin receptors (16). As shown in Fig. 6, the thrombin effect was inhibited by antithrombin III-heparin complex or hirudin, and PPACK-thrombin did not stimulate uPA release from PC-3 cells. This result indicates that intact thrombin is necessary to exert the effect on uPA secretion by PC-3 cells.

**Effect of TRAP on uPA Expression.** To determine whether this thrombin effect is mediated directly by a thrombin receptor or indirectly via other protease actions, we used a new concept for the activation of the thrombin receptor proposed by Vu et al. (11). They cloned a thrombin-specific receptor, which is a member of a seven-transmembrane receptor family, and the presence of this receptor was shown in platelets and endothelial cells. From the deduced amino acid sequence, a novel proteolytic mechanism through which thrombin activates the receptor was proposed. This hypothesis was confirmed by the fact that a synthesized peptide, mimicking the putative new amino terminus which is exposed by proteolysis of an extracellular amino terminal extension of the receptor, could activate thrombin receptors. Based on their report, we synthesized two peptides. One is a new amino terminal peptide, i.e., TRAP, the other is a CP in which the first two amino terminal amino acids were transposed. (The sequence is shown in “Materials and Methods.”)

After 6 h incubation with various concentrations of TRAP and CP, the uPA protein level in the media conditioned by PC-3 cells was measured by ELISA. Fig. 7 shows the effect of TRAP on uPA secretion by PC-3 cells. TRAP produced a dose-dependent increase in uPA secretion. On the other hand, CP had no effect, even at higher concentrations of 50 and 100 μM.

To confirm further the effect of TRAP on uPA expression, we carried out Northern blot analysis (Fig. 8). After 4 h incubation with the peptides, total RNA were extracted and analyzed for uPA mRNA. Again, we observed the increased expression of uPA mRNA by TRAP in a dose-dependent manner but not by CP. These results clearly indicate that PC-3 prostate cancer cells have the same thrombin receptor as endothelial cells and platelets, and the TRAP could bypass the protease action of thrombin to activate the thrombin receptor on PC-3 cells, leading to an increased uPA expression.

**DISCUSSION**

Tumor cells and associated mononuclear phagocytes have been found to possess procoagulant activity (4, 17). These facts imply that thrombin could be generated in the tumor microenvironment through their procoagulant activity. The generated thrombin can convert fibrinogen to fibrin, which helps stromal formation and angiogenesis (18) in tumor tissue, and stimulate tumor cell proliferation; also in the present study, we demonstrated that thrombin could stimulate uPA expression by cancer cells through thrombin-specific receptor activation. Although PC-3 cells are high uPA producers, a further increase in uPA production by thrombin would overwhelm its inhibitors, thus enhancing the breakdown of the extracellular matrix.

Our findings indicate that thrombin showed cell-type specificity. Thrombin enhances uPA production by PC-3 cells but did not affect uPA expression by low or non-uPA producers, DU-145 and LNCaP. A cell type-specific and enhancer-dependent silencer was identified and found to be active in some cell lines which produce little uPA but was not active in the high uPA producer, PC-3 (19). It is assumed that
thrombin does not affect the transcriptional silencer of the uPA gene but stimulates the action of the enhancer. This may be a reason for the cell-type specificity of thrombin effect. Another reason may be that DU-145 and LNCaP cells do not have a thrombin receptor.

In this study, 10 units/ml thrombin caused less uPA expression than 1–2 units/ml thrombin. The reason for this is not fully understood. Thrombin can cleave the extracellular domain of the thrombin receptor at three separate sites, Arg 41, Arg 46, Arg 70, but the activation of the receptor requires only the cleavage at Arg 41, while that of the two other sites does not cause receptor activation. One possibility is that the lower dose of thrombin produced cleavage at Arg 41, resulting in a tethered amino terminus ligand and activation of signal transduction. On the other hand, with a higher dose of 10 units/ml, thrombin may cleave all the three sites including Arg 70, thus releasing the peptide with the amino terminus responsible for receptor activation but with less efficiency than in the former case of the tethered ligand. The free peptide would also diffuse into the extracellular fluid, resulting in a lower concentration on the cell surface.

The presence of thrombin receptor has been demonstrated in platelets and endothelial cells (11). To our knowledge, cancer cells have not been shown to possess a thrombin receptor. In PC-3 cells, TRAP reproduced the same effect as intact thrombin on uPA expression, while CP in which the first two amino acids are transposed had no effect. This successful effect of TRAP indicates that a cancer cell line, PC-3, has a thrombin receptor similar to that on platelets and endothelial cells. Thus, our observations strongly suggest that thrombin generated in the vicinity of cancer cells can modulate cancer cell behavior through stimulation of a thrombin-specific receptor.

Thrombin can convert scuPA to inactive tcuPA; but this phenomenon may not be significant for these reasons: (a) prolonged presence of the active enzyme is unlikely during the time from thrombin receptor stimulation to uPA secretion because of an excess amount of protease inhibitors in plasma and extra cellular fluids; (b) even on the surface of a single cell, the distribution of uPA receptor and of the tissue factor is thought to be different, thus a thrombin generation site could be different from a uPA functioning site; and (c) inactive tcuPA formed by thrombin can be reactivated by dipeptidylaminopeptidase (15). In conclusion, our findings provide a new insight and implica-
tion of the role of thrombin in the regulation of pericellular proteolysis of tumor cells through modulation of uPA expression.

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