Volume 255, number 1, 83–88 FEBS 07573 September 1989

Elastase released from human granulocytes stimulated with *N*-formyl-chemotactic peptide prevents activation of tumor cell prourokinase (pro-uPA)

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Received 28 June 1989

Proteolytic enzymes released from granulocytes upon stimulation with the chemotactic *N*-formyl peptide FNLPNTL (in the presence of cytochalasin B) prevented activation of tumor cell single-chain urokinase-type plasminogen activator (pro-uPA) by plasmin. Elastase was identified by the use of eglin C (elastase inhibitor) and a monoclonal antibody to elastase as the functional proteolytic enzyme in granulocyte supernatants. Action of purified granulocyte elastase on pro-uPA generated enzymatically inactive two-chain uPA linked by disulfide bridges which was indistinguishable by SDS-PAGE from plasmin-generated HMW-uPA. The major elastase cleavage site in pro-uPA was located between Ile^{159} and Ile^{160}; a minor one between Thr^{165} and Thr^{166}. Elastase cannot substitute for plasmin in the proteolytic activation of pro-uPA to enzymatically active HMW-uPA. However, when pro-uPA was first activated by plasmin to form enzymatically active HMW-uPA, this enzymatic activity was not impaired by subsequent elastase treatment.

Prourokinase; Plasminogen activator; Elastase; Granulocyte; Chemotactic peptide; N-terminal amino acid sequence determination

1. INTRODUCTION

Tumor cells produce the urokinase-type plasminogen activator (uPA) as an inactive single-chain precursor molecule (pro-uPA) which is converted to enzymatically active two-chain HMW-uPA by small amounts of plasmin [1,2]. HMW-uPA is a serine protease composed of two polypeptide chains (M_r = 20 000 and 34 000) connected by a disulfide bridge [3]. Conversion of plasminogen to the enzymatically active serine protease plasmin by HMW-uPA is a general mechanism which initiates pericellular proteolysis [1]. Plasminogen activation by HMW-uPA is involved in tissue and stroma degradation in a number of benign as well as malignant conditions [1]. Recent studies demonstrated that HMW-uPA is a potent chemotactic factor for granulocytes in vivo in addition to tumor stroma breakdown products which may also attract granulocytes into the tumor tissue [4]. Phagocytic cells have been identified by immunohistochemistry in tumors of the uterine cervix [5]. Granulocytes contain enzymatically active proteases: elastase, collagenase, and gelatinase, which are released by chemotatic activation [6]. Each of these proteases is able to attack components of the extracellular matrix [6]. We now report that granulocyte elastase, released upon stimulation with chemotactic peptides, degrades tumor pro-uPA to an enzymatically inactive uPA form which

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Abbreviations: uPA, urokinase-type of plasminogen activator; pro-uPA, single-chain proenzyme form of urokinase; HMW-uPA, two-chain form of high molecular weight urokinase; BSA, bovine serum albumin

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/89/$3.50

cannot be converted to enzymatically active HMW-uPA by plasmin.

2. MATERIALS AND METHODS

2.1. *Materials*

Reagents were obtained from the sources indicated in parentheses. Pro-uPA (spec. act. of 135 000 U/mg) produced by kidney tumor cell line TCL 598 (Sandoz, Nürnberg, FRG). HMW-uPA (Serono, Freiburg, FRG). Human granulocyte elastase (Protogen, Läufelfingen, Switzerland). Human plasmin and fibrinogen (KabiVitrum, München). Prestained gel standards (Pharmacia, Freiburg, FRG). Aprotinin and test-thrombin (Behring Werke, Marburg, FRG). Bovine serum albumin (BSA) and cytochalasin B (Sigma, München, FRG). *N*-formyl-Nle-Leu-Phe-Nle-Tyr-Lys (FNLPNTL) and substrates S-2444 and methoxysuccinyl-Ala-Ala-Pro-Val-paranitroanilide (Bachem, Hannover, FRG). Eglin C was donated by Dr H. Fritz, Ludwig Maximilians University, München, FRG, and murine monoclonal antibody to human granulocyte elastase by Dr T. Cotter, Maynooth, Ireland. All other reagents were of analytical grade.

2.2. *Methods*

2.2.1. SDS-Page

SDS-PAGE was performed according to Laemmli [7] applying 15% acrylamide gels. Prestained proteins served as molecular weight markers. Samples were reduced by 10 mM 2-mercaptoethanol. Proteins were stained with Coomassie blue R-250 (Serva, Heidelberg, FRG) and destained with 7% acetic acid.

2.2.2. Assays of enzymatic activity of HMW-uPA

Amidolytic activity of HMW-uPA was measured in 0.1 M Tris-HCl, pH 8.8, containing 0.1 M NaCl and 0.01% BSA using the synthetic substrate S-2444 [8]. Fibrin clot lysis assay was performed according to Kruithof et al. [9] using fibrin gels which contain excess of plasminogen. Lysis volume in tubes ($r = 5$ mm) was calculated in % of total gel volume.

2.2.3. Purification of human peripheral blood granulocytes

Granulocytes were purified according to Boyum [10]. Viability was >98% as judged by trypan blue exclusion. Contaminating erythrocytes were lysed by short exposure (5 min) of cells to lysis buffer (160 mM NH_4Cl, 12 mM $NaHCO_3$, 0.1 mM EDTA, pH 7.3). Cells were stored at room temperature in PBS-2% BSA without calcium and magnesium salts.

2.2.4. Degranulation of granulocytes by stimulation with the *N*-formyl chemotactic peptide FNLPNTL in the presence of cytochalasin B

5×10^6 granulocytes were incubated in 1 ml PBS (containing 0.5 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1% BSA) with 5 μg cytochalasin B (5 min, 37°C). 10 μl of 10^{-6} M FNLPNTL was added (30 min, 37°C) and supernatants collected by low speed centrifugation ($250 \times g$, 5 min). Elastase activity was assessed by using methoxysuccinyl-Ala-Ala-Pro-Val-paranitroanilide as the human elastase specific substrate [11].

2.2.5. Identification of proteolytic cleavage site in pro-uPA by sequence analysis

370 μg of pro-uPA in 1 ml PBS were digested with 20 μg (0.5 U) of purified granulocyte elastase in 200 μl of 62 mM Tris-HCl, pH 7.5 (30 min, 37°C). Digestion of pro-uPA into two-chain uPA was verified by SDS-PAGE. The digested sample and an undigested control sample were dialyzed against 3% acetic acid. An aliquot of each sample was sequenced by Edman degradation in a prototype spinning-cup sequenator [12]. The amino acid derivatives were identified by isocratic HPLC analysis.

3. RESULTS

Supernatants of human granulocytes, which had been stimulated with the *N*-formyl chemotactic peptide FNLPNTL, contained proteases which

Table 1

Identification of elastase in supernatants of stimulated human granulocytes as the functional protease to degrade tumor cell pro-uPA

Stimulus	Assay	
	Fibrin clot lysis (ml)	Amidolytic activity (U/ml)
PBS	0.47 ± 0.018	16.0 ± 2.4
+ Cytochalasin B	0.49 ± 0.065	14.6 ± 4.6
+ Cytochalasin B and FNLPNTL	0.06 ± 0.012	2.1 ± 1.1
+ Cytochalasin B and FNLPNTL, then addition of eglin C	0.48 ± 0.015	21.0 ± 2.7
+ Cytochalasin B and FNLPNTL, then adition of antibody to elastase	0.47 ± 0.015	15.8 ± 2.8

5×10^6 granulocytes/ml PBS containing 0.5 mM $MgCl_2$, 1 mM $CaCl_2$, and 0.1% BSA were pretreated with 5 μg cytochalasin B/ml (5 min, 37°C) prior to the addition of 10^{-8} M FNLPNTL (30 min, 37°C). Supernatants were collected by low speed centrifugation and then incubated with eglin C (12 μM) or monoclonal antibody to elastase (15 μM) for 30 min, 37°C. Cells which had been treated with buffer or cytochalasin B, only, served as controls. Aliquots of 30 μl or pro-uPA (500 ng) were mixed with 30 μl of the various cell supernatants (30 min, 37°C) and then subjected to fibrin clot assay as described in section 2. For assay of amidolytic activity with substrate S-2444, 0.3 nM of plasmin was added. The reaction was stopped by addition of 200 KIU aprotinin/ml. Average values obtained from 4 different donors are expressed ± standard deviation

prevented conversion of tumor cell pro-uPA to enzymatically active HMW-uPA (table 1). When pro-uPA was treated with supernatants from granulocytes, which were stimulated with 10^{-8} M FNLPNTL in the presence of cytochalasin B, pro-uPA was impaired in such a way that the subsequent treatment with plasmin did not result in an enzymatically active HMW-uPA. Preincubation of cell supernatants, however, with eglin C (inhibitor for elastase and cathepsin G [13]) or an antibody to the enzymatically active region of elastase, prevented the inactivation of pro-uPA by the cell supernatant. Thus by these inhibition experiments the proteolytic activity in granulocyte supernatants was identified as elastase.

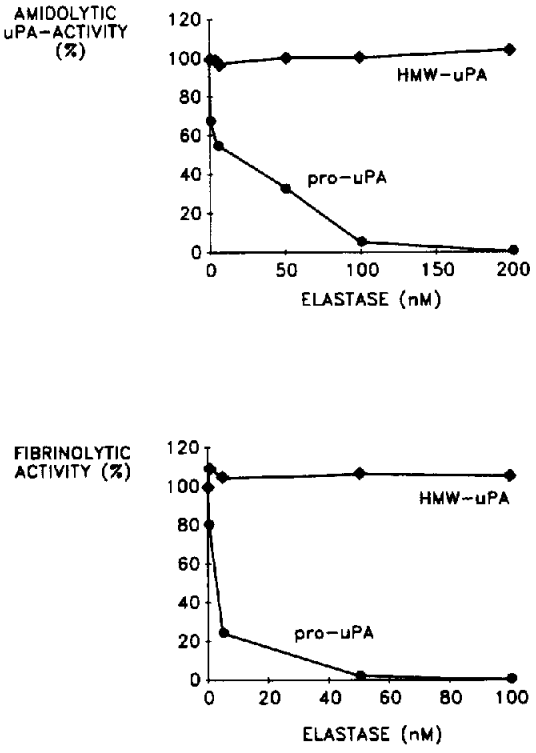

Fig.1. Effect of purified granulocyte elastase on pro-uPA and HMW-uPA. Elastase (0–200 nM in PBS containing 0.5 mM $MgCl_2$, 1 mM $CaCl_2$, and 0.1% BSA) was incubated with 370 ng HMW-uPA/50 μl (30 min, 37°C) and then treated with 0.13 nM plasmin (45 min, 37°C). Plasmin reaction was stopped by the addition of 200 KIU aprotinin/ml. Amidolytic activity was measured as described in section 2. For fibrinolytic assay elastase (0–200 nM) was incubated with 500 ng pro-uPA as described for the amidolytic assay and then layered on top of fibrin gels as described in section 2. Values are representative for cell supernatants collected from 3 different donors.

Exposure of pro-uPA to purified granulocyte elastase (0 – 6 μg/ml, 30 min, 37°C) did not result in an enzymatically active HMW-uPA. Hence, elastase cannot substitute for plasmin in pro-uPA activation in vitro. Moreover, human elastase at a protein weight ratio of 1.5:10 for elastase to pro-uPA was sufficient to prevent activation of pro-uPA in a way that subsequent plasmin treatment did not generate enzymatically active HMW-uPA (fig.1). This confirms the results obtained with the

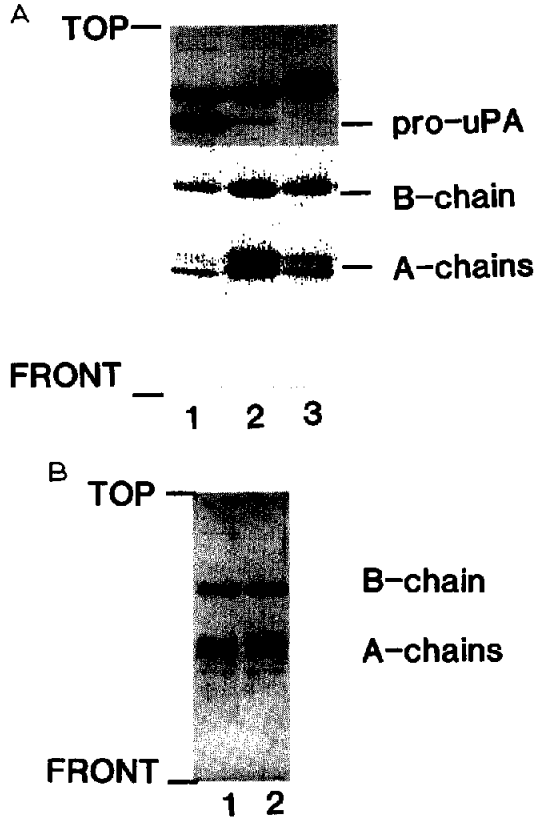

Fig. 2. SDS-PAGE of pro-uPA and HMW-uPA treated with elastase or plasmin. 5 μg of pro-uPA (A) and HMW-uPA (B) were treated with 0.13 μg of plasmin or 0.37 μg of purified granulocyte elastase in 30 μl of PBS for 30 min, 37°C, and then heated with 20 μl sample buffer containing 2-mercaptoethanol for 5 min, 100°C. Polypeptide chains were separated by SDS-PAGE (15% acrylamide) and stained with Coomassie blue R-250. Please, note that the prominent band above the B-chain is not part of pro-uPA. This band does not stain with antibodies to uPA in Western blots and represents albumin which was added to pro-uPA by the manufacturer as a stabilizing protein. (A) pro-uPa was treated with buffer (lane 1); elastase (lane 2); plasmin (lane 3). (B) HMW-uPA was treated with buffer (lane 1); elastase (lane 2).

Table 2
Identification of granulocyte elastase cleavage sites in pro-uPA by N-terminal amino acid sequence determination

Sample	N-terminal sequence
pro-uPA	1 5 10 15 Ser-Asn-Glu-Leu-His-Gln-Val-Pro-Ser-Asn-Cys-Asp-Cys-Leu-Asn-Gly-
Elastase-treated pro-uPA	1 5 10 15 Ser-Asn-Glu-Leu-His-Gln-Val-Pro-Ser-Asn-Cys-Asp-Cys-Leu-Asn-Gly-
	160 165 170 175 Ile-Gly-Gly-Glu-Phe-Thr-Thr-Ile-Glu-Asn-Gln-Pro-Trp-Phe-Ala-Ala-
	166 170 175 180 Thr-Ile-Glu-Asn-Gln-Pro-Trp-Phe-Ala-Ala-Ile-Tyr-Arg-Arg-His-Arg-

A sample of 370 μg pro-uPA, dissolved in 1 ml PBS, was incubated with 20 μg (0.5 U) of granulocyte elastase (30 min, 37°C), acidified with acetic acid to pH 3 and then dialyzed extensively against 3% acetic acid. Undigested pro-uPA was also acidified. An aliquot each of the samples (about 1 nmol) was subjected to sequence analysis for 16 cycles. The sequence of human serum albumin, included as a stabilizer in the uPA preparation, was also clearly observed in both samples (data not shown).

supernatants of stimulated granulocytes. Enzymatically active HMW-uPA (obtained from pro-uPA by plasmin treatment or the commercially available HMW-uPA purified from urine) was not appreciably affected by subsequent elastase treatment. Human granulocytes also produce pro-uPA which is stored in specific granules [14]. However, under the conditions applied (short-term stimulation) no significant release of pro-uPA into cell supernatants of stimulated granulocytes was detected by ELISA or enzymatic assays (data not shown).

Proteolytic action of elastase on pro-UPA was verified by SDS-PAGE (fig.2A). Similar to plasmin, elastase splits pro-uPA into two chains of M_r = 34 000 (B-chain) and 20 000 (A-chain). When HMW-uPA was treated with the same concentrations of plasmin or elastase as described for pro-uPA, no significant degradation of the polypeptide chains was observed (fig.2B). At a high concentration of elastase (1 μM), however, significant breakdown of the A-chain as well as the B-chain was observed (data not shown).

The elastase cleavage site in pro-uPA was evaluated by N-terminal amino acid sequence determination. The unfractionated digest was analyzed in parallel with untreated pro-uPA (table 2). In the undigested pro-uPA the known sequence (16 amino acids) [3] was found. In the elastase-treated pro-uPA two additional N-terminals were detected. The prominent cleavage site is between Ile^{159} and Ile^{160}, the second minor one between Thr^{165} and Thr^{166} (approximate ratio of 5:1). The new sequences started with Ile^{160} and Thr^{166}, respectively. This is one and seven residues behind the beginning of the plasmin-derived B-chain.

4. DISCUSSION

Human phagocytic cells are mediators of tissue-destroying events in inflammation and tumor spread [6]. These processes are linked to the phagocyte's ability to release toxic agents or enzymes upon receptor-mediated stimulation which can dissolve extracellular matrix components and destroy cells. More than 50 of such compounds have been identified including the proteases elastase, collagenase, and gelatinase [6]. Chemotactically attracted phagocytic cells are found in the tumor tissue and the stroma surrounding the tumor nests [5]. Tumor cells secrete the proenzyme form pro-uPA of the urokinase-type plasminogen activator [1,2].

The conversion of pro-uPA to enzymatically active HMW-uPA is limited to the specificity of the enzyme applied. Plasmin converts pro-uPA into enzymatically active HMW-uPA [1–3]. Degradation of the tumor stroma is initiated by plasmin which is generated from plasminogen by the action of HMW-uPA [1]. Trypsin and plasma kallikrein may substitute for plasmin [15]. Proteolytic action of thrombin [15,16] and as shown in the present

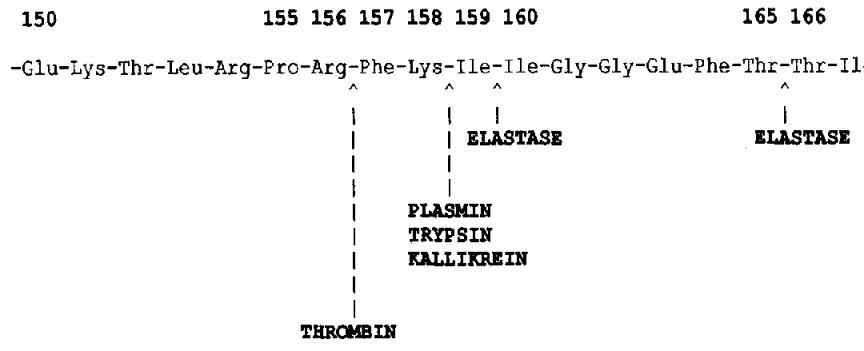

Fig. 3. Proteolytic cleavage sites within the pro-uPA molecule.

report, granulocyte elastase, on pro-uPA results in enzymatically inactive uPA-forms which are resistant to subsequent activation by plasmin. Active and inactive forms of uPA were indistinguishable by SDS-PAGE. Plasmin and trypsin specifically cleave the Lys^{158}- Ile^{159} peptide bond within the single chain pro-uPA [15]. Thrombin specifically cleaves the Arg^{156}-Phe^{157} peptide bond which is located two amino acid residues prior to this bond [15] (fig.3).

In the present work evidence is presented that human granulocyte elastase specifically cleaves pro-uPA at the peptide bonds Ile^{159}-Ile^{160} and Thr^{165}-Thr^{166}, the former cleavage site being predominant (table 2; fig.3). These cleavage sites are located just behind the plasmin-cleavage site and yield A- and B-chains of similar length as shown on SDS-PAGE for plasmin degradation. Cleavage of pro-uPA within the disulfide-linked domain by proteolytic enzymes will cause a conformational change within the molecule [15–17]. Depending on which peptide bond is cleaved, an enzymatically active or inactive two-chain molecule is generated. Elastase was identified as the functional enzyme in cell supernatants of chemotactically activated human granulocytes, as both, eglin C and a monoclonal antibody to elastase, blocked proteolysis of pro-uPA by these cell supernatants. Heiple and Ossowski [14] also reported on the destructive potential of cell supernatants of stimulated granulocytes on pro-uPA without identifying the components involved. The inactivation capacity of these enzymes was inhibited by the serine protease inhibitor diisopropylfluorophosphate (DFP). Heiple's and our results support the notion that granulocyte elastase is one of the key enzymes which may inactivate pro-uPA released by cells.

Elastase content in phagocytic cells can be identified by immunohistochemistry within the tumor stroma and close to tumor cells of breast cancer patients (Schmitt and Jänicke, unpublished). Depending on which enzyme, elastase or plasmin, cleaves pro-uPA, enzymatically inactive uPA or active HMW-uPA will be generated. Interestingly, enzymatically active HMW-uPA (which has been formed by plasmin treatment of pro-uPA) is not inactivated by subsequent elastase [17] or thrombin [16] action.

Although elastase does not inactivate HMW-uPA, the enzymatic activity of HMW-uPA in solution may be inhibited by the specific plasminogen activator inhibitors PAI-1 and PAI-2 [1,2]. Therefore, separate mechanisms appear to exist which regulate pro-uPA conversion and HMW-uPA activity: inactivation of (i) pro-uPA by granulocyte elastase or thrombin and (ii) HMW-uPA by plasminogen activator inhibitors PAI-1 or PAI-2. HMW-uPA bound to receptors on tumor cells is supposed to be involved in tumor growth, malignant invasion, degradation of the tumor stroma and metastasis [1,2]. Inactivation of pro-uPA by granulocyte elastase, in addition to inactivation of HMW-uPA by PAI-1/2, therefore may delay the proteolytic processes in tumor-associated fibrinolysis [18].

Acknowledgements: This work was supported by Sonderforschungsbereich 207 (Project G10) of the Deutsche Forschungsgemeinschaft and a grant of the Wilhelm Sander-Stiftung. Dr Kanayama is a visiting investigator supported by the Japanese Educational Ministry.

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