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Inactivation of Human Tumor Cell Pro-urokinase by Granulocyte Elastase

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Supernatant obtained from granulocytes stimulated in the presence of cytochalasin B by the chemotactic peptide N-formyl-norleucyl-leucyl-phenylalanyl-norleucyl-tyrosyl-lysine displayed an inhibitory effect on the plasmin-dependent conversion of tumor urokinase-type plasminogen activator proenzyme (pro-uPA) to the active form of uPA. Moreover, the supernatant was also found to inhibit the fibrinolytic activity of human vulva (A431) and breast (MCF7) carcinoma cell lines, which contain large amounts of pro-uPA, by 87% and 96%, respectively. By using eglin C (elastase inhibitor) and a monoclonal antibody to elastase (proteolytic activity blocker of the enzyme), elastase was identified as the key enzyme of the supernatant in these phenomena. Purified elastase converted pro-uPA to an enzymatically inactive molecule composed of two polypeptide chains of M_r=33,000 and 22,000 linked to each other by a disulfide bond. Elastase-containing granulocytes were identified by immunohistochemistry techniques in the tissues of squamous cell carcinoma and adenocarcinoma of uterus. The cells were found close to the tumor cells and in the stroma surrounding the tumor nests. By immunohistochemical staining, uPA was also found in the tumor cells. Evidently, elastase released by chemotactically activated granulocytes, which are attracted into tumor tissues, may inhibit the conversion of pro-uPA to enzymatically active uPA in the tumor cells.

Key words: Granulocyte elastase — Pro-urokinase — Fibrinolysis — Breast cancer — Uterine cancer

Plasminogen activators are major mediators for pericellular proteolysis. In tissues of breast, prostate, cervix, and colon cancer, urokinase-type plasminogen activator (uPA) is produced and secreted as an enzymatically inactive single-chain proenzyme form (pro-uPA).[1-3] The increase of uPA in tumor cells has been observed, associated with increased tumor growth and metastatic potential.[4] Pro-uPA may be converted by small amounts of plasmin into the enzymatically active two-chain form uPA (HMW-uPA) which subsequently converts plasminogen into the broad-spectrum serine protease plasmin.[5] Plasmin degrades the finbrin-fibronectin matrix of the tumor stroma, thus releasing fibrin remnants and cross-linked fibrin-fibronectin compounds.[6, 7]

In solution the naturally occurring plasminogen activator inhibitors PAI-1 and PAI-2 may inactivate uPA, but do not bind to pro-uPA.[8, 9] Here we report that elastase released by chemotactically activated human granulocytes inactivates and degrades tumor cell pro-uPA.

The abbreviations used are: APAAP, alkaline phosphatase anti alkaline phosphatase complex; BSA, bovine serum albumin; CHO-NLPNTL, CHO-norleucyl-leucyl-phenylalanyl-norleucyl-tyrosinyl-lysine; HMW-uPA, enzymatically active high-molecular-weight two chain form of urokinase-type plasminogen activator; moAB, monoclonal antibody; PAP, peroxidase anti peroxidase complex; pro-uPA, proenzyme form of the urokinase-type plasminogen activator; S-2444, pyro-Glu-Gly-Arg-*p*-nitroanilide; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TBS, Tris-buffered saline, pH 8.5; TMB, 3,3′,5,5′-tetramethylbenzidine.

MATERIALS AND METHODS

Reagents were purchased from the sources indicated in parenthesis. Pro-uPA from kidney tumor cell line TCL 598 (specific activity 135,000 units/mg, purity: 98%) (SANDOZ, FRG); purified human granulocyte elastase (purity: 99%) (Protogen, Switzerland); HMW-uPA (Mochida, Tokyo); aprotinin, test thrombin (Behringwerke, FRG); human fibrinogen, human plasmin, and chromogenic substrate S-2444 (KabiVitrum, Sweden); dextran T 500, prestained SDS-PAGE standards (Pharmacia, Sweden); cytochalasin B, rabbit peroxidase-antiperoxidase complex (PAP), and levamisole (Sigma, USA); CHO-Nle-Leu-Phe-Nle-Tyr-Lys (FNLPNTL) and methoxysuccinyl-Ala-Ala-Pro-Val-*para*-nitroanilide (Bachem, FRG); mouse anti-rabbit-IgG, mouse monoclonal antibody to human granulocyte elastase (clone M752), and alkaline phosphatase anti-alkaline phosphatase complex (APAAP) (Dakopatts, Kyoto) monoclonal antibody #377 and #394 to human uPA (American Diagnostics, USA); peroxidase-conjugated avidin, and 3,3′,5,5′-tetramethylbenzidine (TMB) (Sigma). Eglin C was a gift from Dr. H. Fritz (FRG).

Rabbit-anti human granulocyte elastase was prepared by ourselves.[10]

Breast cancer cell line MCF7 and vulva cancer cell line A431 cells were supplied by American Type Culture Collection (USA). The cells were grown in RPMI 1640 medium supplemented with L-glutamine and 5% fetal calf serum (FCS) at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Cultures initiated at a density of 5×10^4 cells/ml grew exponentially to about 1×10^6 cells/ml in 3 days. In all the experiments described here 75-cm^2 tissue culture flasks were inoculated with 30 ml of cell suspension containing $1–3\times10^5$ cells/ml. All treatments were carried out on exponentially growing cell cultures. Cell viability was assessed by trypan blue exclusion.

Tissue preparation Specimens from patients with uterine cervical and endometrial carcinoma were fixed in buffered formalin and then embedded in paraffin. Sections were cut and mounted on microscope slides. These paraffin-embedded sections were dewaxed in Roti-Histol (a xylene substitute), followed by isopropanol and 96% (v/v) ethanol, and then rehydrated in phosphate-buffered saline (PBS).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) pro-uPA (5 μg) was incubated with 20 μl of 50 m*M* Tris-HCl, pH 7.3, containing 0.45 μg of elastase (final concentration 500 n*M*) for 30 min at 37°C. The reaction mixture was subjected to SDS-PAGE according to Laemmli[11] using 15% polyacrylamide gels. Samples were heated (5 min, 100°C) in the presence of 10 m*M* 2-mercaptoethanol. Proteins were stained with Coomassie Blue R-250 and destained with 7% acetic acid. HMW-uPA (5 μg) was also subjected to SDS-PAGE.

Enzymatic assays of uPA and elastase uPA activity: To determine the effect of elastase on pro-uPA, 50 units of pro-uPA/50 μl of 50 m*M* Tris-HCl, pH 7.3, was incubated with various concentrations of elastase (0.5–200 n*M*) for 30 min at 37°C. The mixture will be referred to as elastase-treated pro-uPA. To convert the pro-uPA preparations to uPA, the samples were subsequently incubated with 2.8 n*M* plasmin (45 min, 37°C). Plasmin action was stopped by the addition of 200 kIU/ml aprotinin.

Samples containing elastase (0–200 n*M*) in Tris-HCl (in the absence of pro-uPA) with added plasmin served as controls.

The enzymatic activity of uPA was measured with 0.33 m*M* chromogenic substrate S-2444. Enzyme activity was determined by measuring the change in absorbance at 405 n*M*. The change of absorbance produced by 50 units pro-uPA/50 μl Tris-HCl which had been treated with 2.8 n*M* plasmin (45 min, 37°C) was defined as 100% HMW-uPA activity.

Fibrin-clot lysis assay was performed essentially according to Kruithof *et al.*[12] Human plasmin (ogen)-rich fibrinogen (1.5 mg/ml) and dextran (1.5 mg/ml) were dissolved at room temperature in 0.2 *M* Tris-HCl, pH 7.8. Thrombin (30 units/50 m*M* imidazole-140 m*M* NaCl, pH 7.35) was diluted twenty-fold in 50 n*M* $CaCl_2$, then 1.2 ml of fibrinogen-dextran was mixed in a glass tube with 0.4 ml of the thrombin-calcium solution. Fibrin gels formed were allowed to stabilize for at least 30 min prior to use.

Elastase-treated pro-uPA samples were placed on top of the fibrin gels in the tube. The tubes were incubated (2 h, 37°C). Lysis volume was calculated from the height of the dissolved gel. Lysis volume caused by 50 units of pro-uPA/50 μl Tris-HCl was defined as 100% fibrinolytic activity.

Elastase activity: The enzymatic activity of elastase was measured with 0.67 m*M* chromogenic amidolytic substrate, methoxysuccinyl-Ala-Ala-Pro-Val-*para*-nitroanilide, in 0.1 *M* Tris-HCl, pH 8.3, containing 960 m*M* NaCl. Enzymatic activity was determined by measuring the change in absorbance at 405 nm. Purified granulocyte elastase (0–0.25 U/ml) served as a 100% activity.

Determination of elastase and uPA by ELISA Elastase: The wells of a microtiter plate were coated with 100 μl of a solution containing moAB to elastase (0.4 μg/ml) in 50 m*M* bicarbonate buffer, pH 9.6 (4°C, 16 h), and then blocked with TBS-2% bovine serum albumin (BSA) (1 h, 37°C). Granulocyte supernatant (100 μl) containing 10 m*M* phenylmethanesulfonyl fluoride in TBS-2% BSA was added and the plate was incubated (1 h, 23°C). The plate was washed in PBS, then 100 μl per well of rabbit-IgG anti-elastase (4 μg/ml) in TBS-0.05% Tween 20 was added and incubation was continued (1 h, 23°C). Subsequently, after additional washing steps, mouse-IgG-anti rabbit-IgG was added to the wells (1 h, 23°C), and then rabbit-PAP was added (1 h, 23°C). After washing steps, 100 μl of substrate solution was added (0.1 *M* sodium acetate-citrate buffer, pH 6.0, containing 42 n*M* TMB and 0.0045% H_2O_2). The reaction was terminated by addition of 50 μl of 1 *M* H_2SO_4/well and the optical density was read at 450 n*M* with a Bio Rad EIA reader (USA).

Pro-uPA and uPA: Wells of a microtiter plate were coated with 100 μl of a solution containing moAB #394 (1.5 μg/ml in 50 m*M* bicarbonate buffer, pH 9.6 (16 h, 4°C) and then blocked with TBS-2% BSA (1 h, 37°C). After adding the sample (100 μl in TBS-2% BSA-0.05% Tween 20, 16 h, 4°C) biotinylated moAB #377 to uPA was added (1 h, 23°C). After washing, avidin-peroxidase in 20 m*M* Tris-HCl-0.5*M* NaCl-0.05% Tween 20, pH 8.5, was added (1 h, 23°C). Peroxidase activity was detected by using the substrate solution described for the elastase-ELISA. The uPA-ELISA detects pro-uPA and

the proteolytically degraded forms of uPA as well (Hollrieder, unpublished results). Detection limit: 40 pg of uPA or pro-uPA/ml.

Stimulation of human peripheral blood granulocytes by N-formyl chemotactic peptide CHO-NLPNTL Granulocytes were isolated essentially as described.[13] Briefly, 50 ml of fresh human blood obtained from four healthy donors was anticoagulated with 5,000 units of heparin and immediately centrifuged through Ficoll-Hypaque. The cell pellet was resuspended and residual erythrocytes were lysed by using 0.16 *M* NH_4Cl containing 12 m*M* $NaHCO_3$, 0.1 m*M* EDTA, pH 7.3. Granulocytes were washed with PBS. Granulocytes (5×10^6) in 1 ml of PBS containing 0.1% BSA, 1 mg glucose, 1 m*M* $CaCl_2$, and 0.5 m*M* $MgCl_2$, pH 7.3, were pretreated with cytochalasin B (5 μl/ml) for 5 min at 37°C. Then 10^{-8} *M* FNLPNTL was added and the cells were incubated for 30 min at 37°C. After low-speed centrifugation (250*g*, 5 min), the supernatants were collected and then stored at −20°C until measurement.

Fibrinolytic activity of cancer cells Ten μl of A431 or MCF7 cells (2×10^6) in PBS, pH 7.3 were preincubated with 40 μl of PBS, supernatant from non-activated granulocytes or supernatant from activated granulocytes (30 min, 37°C). To avoid the influence of myeloperoxidase on cancer cells, 0.25 m*M* sodium azide was added to the reaction mixture. After low-speed centrifugation (250*g*), the pellet was collected and resuspended in 10 μl of PBS. Each sample was placed on top of the fibrin gel in the tube (8 h, 37°C). After incubation, lysis volume was calculated as described before. This measurement was performed in triplicate.

To determine the uPA content of A431 or MCF7 cells, 2×10^6 cells were incubated with 0.1% Triton X in Tris buffer, pH 7.3 (12 h, 4°C). After treatment, uPA activity and content were measured as above.

Immunohistochemical detection of uPA and elastase Dewaxed paraffin-embedded tissue sections of uterine squamous and adenocarcinoma were processed for the detection of uPA and elastase. The slides were rinsed in 50 m*M* Tris-HCl containing 125 m*M* NaCl, pH 8.1 (TBS) and covered with 20% rabbit serum (20 min, 23°C). Subsequently, a 1:50 dilution of monoclonal antibody to uPA (#394) or elastase was added in TBS. After being washed in TBS, the sections were reacted with a 1:50 dilution of Ig rabbit anti mouse Ig in TBS (30 min, 23°C), and again washed with TBS. Then a 1:50 dilution of mouse-APAAP in TBS was added (30 min, 23°C). The sections were washed in TBS, and the alkaline phosphatase-dependent staining was developed with 0.2 mg/ml Naphthol-AS-MX phosphate in combination with 10 mg/ml Fast Red TR in 0.2 *M* Tris-HCl (pH 8.5) containing 1 m*M* levamisole to block intrinsic alkaline phosphatase activity (20 min, 23°C). The tissue sections were washed in TBS and water and mounted in glycerol-gelatin. Controls were performed by omitting the first antibody or by replacing the first antibody with irrelevant IgG-antibodies of the relevant species.

RESULTS

Degradation of pro-uPA by purified human granulocyte elastase To test the effect of elastase on tumor pro-uPA *in vitro*, elastase was incubated with purified pro-uPA and then the samples were subjected to SDS-PAGE. Elastase degraded pro-uPA (M_r=55,000) into a molecule consisting of two major polypeptide chains of M_r=33,000 and M_r=22,000 connected by a disulfide bond(s) (Fig. 1). In non-reduced condition, this molecule produced by elastase treatment gave a single band of M_r=55,000 (data not shown). Two bands of A chains were observed. The lower band of A chain is thought to be a molecule which lacks the epidermal growth factor (EGF) domain of pro-uPA. The EGF domain of pro-uPA can be split off easily.

Effect of elastase on pro-uPA activation The elastase-treated samples were then subjected to plasmin treatment and tested for amidolytic or fibrinolytic activity. The new finding is that elastase treatment of pro-uPA inhibited

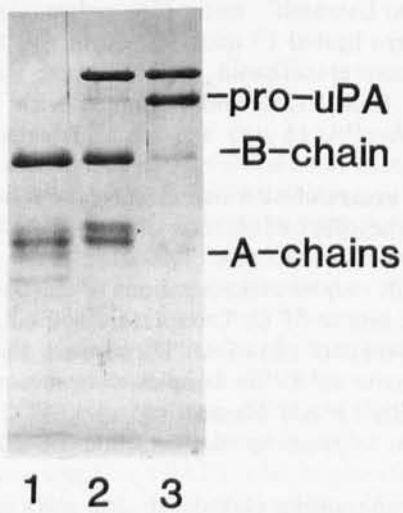
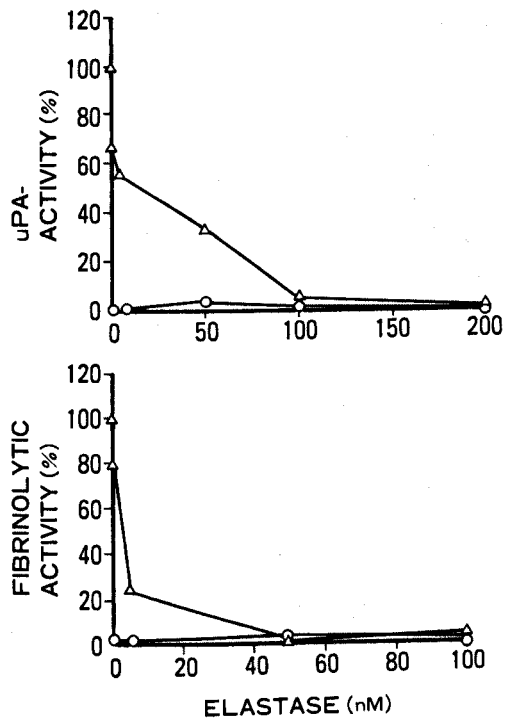


Fig. 1. SDS-PAGE analysis (15% acrylamide, reducing conditions) of pro-uPA treated with proteases. Five μg of pro-uPA in 30 μl of TBS was treated with elastase (30 min, 37°C). Lane 1: 5 μg of HMW-uPA. Lane 2: Pro-uPA treated with 0.45 μg of elastase. Line 3: Pro-uPA. The action of the elastase on pro-uPA produced polypeptide chains of similar molecular mass (A-chain(s), M_r 22,000; B-chain, M_r 33,000). The prominent stained polypeptide band above pro-uPA represents albumin which was added by the manufacturer to the pro-uPA preparation as a stabilizing agent.

the conversion of pro-uPA by the subsequent addition of plasmin. Elastase has been discovered to digest plasminogen into a smaller fragment (mini-plasminogen). The fragment contains the active site of plasmin and still has the properties of plasmin, especially amidolytic and fibrinolytic activities.[14] Thus, we assume that elastase has no effect on the active site of plasmin.



Therefore, inhibition of the conversion of pro-uPA to active uPA should be the direct effect of elastase on pro-uPA. The potential of pro-uPA to be converted by plasmin into an enzymatically active uPA-molecule (latent activity) decreased with increasing elastase concentration prior to plasmin treatment (Fig. 2). When pro-uPA was treated with elastase first, and then plasmin was added, the enzymatic activity of pro-uPA was completely inhibited at 100 n*M* elastase. Even at a concentration as low as 0.5 n*M* elastase, a loss of 35% of the activity was observed. When pro-uPA was incubated with plasmin first, and then elastase (0–200 n*M*) was added, no loss of pro-uPA activity was detected (data not shown). Samples containing elastase and plasmin did not dissolve a fibrin clot.

Identification of elastase as the functional protease in granulocyte supernatant When human peripheral blood granulocytes were stimulated in suspension with 10^{-8} *M* chemotactic peptide CHO-NLPNTL, a significant

Fig. 2. Effect of elastase on latent amidolytic and fibrinolytic activity of pro-uPA. For determination of latent amidolytic activity (upper plot) of pro-uPA in the presence of proteases, pro-uPA (50 units) in 50 m*M* Tris-HCl, pH 7.3, was incubated with elastase (30 min, 37°C), and then 2.8 n*M* plasmin was added (45 min, 37°C) (△). Fifty units of pro-uPA treated with plasmin in Tris-HCl in the absence of elastase was defined as 100% amidolytic activity. Elastase (75 min, 37°C) (○). For determination of fibrinolytic activity (lower plot) of pro-uPA in the presence of proteases, pro-uPA (50 units) in 50 m*M* Tris-HCl, pH 7.3, was incubated with elastase (30 min, 37°C), and then applied to the fibrin gels in glass tubes (2 h, 37°C) (△). Elastase only (○) (75 min, 37°C). Lysis caused by 50 units of pro-uPA in Tris-HCl without elastase was defined as 100% fibrinolytic activity.

Table I. Detection of Elastase and uPA in Supernatants Released by Granulocytes Stimulated with the Chemotactic Peptide CHO-NLPNTL

Reagent	Elastase activity (U/ml)	Elastase antigen (μg/ml)	uPA activity (U/ml)	uPA antigen (ng/ml)
Buffer	ND[a)]	0.02±0.02	ND	0.29±0.11
5 μg cytochalasin B/ml	0.016±0.01	3.2±3.3	ND	0.21±0.03
5 μg cytochalasin B/ml +10^{-8} *M* CHO-NLPNTL	0.26±0.01	62.5±48.7	ND	0.28±0.07

a) Not detected.
Granulocytes (5×10^6/ml) were preincubated with cytochalasin B (5 μg/ml) and then stimulated with 10^{-8} *M* CHO-NLPNTL as described in the "Materials and Methods" section. The cell supernatants were assayed for elastase activity with the substrate methoxysuccinyl-Ala-Ala-Pro-Val-*para*-nitroanilide, and for uPA activity with the substrate S-2444. Elastase and uPA antigens were measured by ELISA. Mean values (n=4) are given with the standard deviation.
Note: Human granulocytes contain uPA.[17] Although there is a significant release of enzymatically active granulocyte elastase upon stimulation, no significant release of uPA by granulocytes under the conditions applied was detected by ELISA or enzymatic assay.

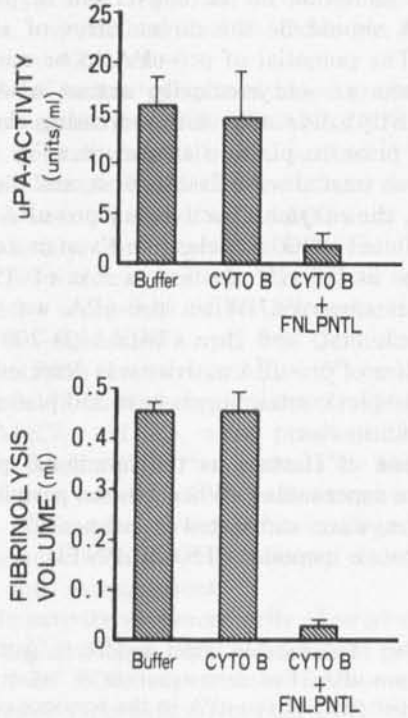


Fig. 3. Effect of granulocyte supernatant on pro-uPA. Determination of amidolytic (upper plot) and fibrinolytic (lower plot) activity. Supernatants (60 μl) from granulocytes treated with the reagents indicated were mixed with 100 units of pro-uPA contained in 60 μl of PBS (30 min, 37°C). Aliquots (60 μl) were applied to a fibrin clot. Other aliquots (60 μl) were treated with 2.8 n*M* plasmin for 45 min at 37°C and subsequently amidolytic activity was measured with substrate S-2444. Untreated HMW-uPA (prepared from pro-uPA by plasmin treatment) served as the control.

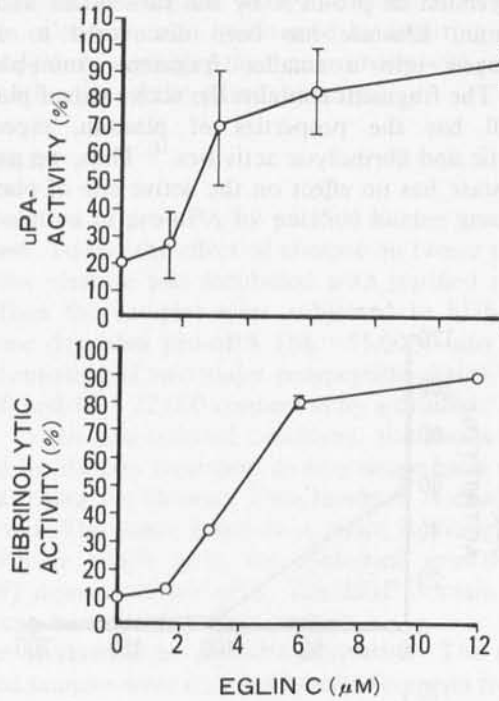


Fig. 4. Inhibition of elastase activity in granulocyte supernatant by eglin C. Supernatant (60 μl) from granulocyte stimulated with 10^{-8} *M* CHO-NLPNTL in the presence of cytochalasin B (for details see "Materials and Methods" section) were incubated with different concentrations of eglin C (0–12 μM) and then mixed with 50 units of pro-uPA in 60 μl of PBS (30 min, 37°C). Half the volume of the reaction mixture was applied to fibrin clots in tubes (2 h, 37°C) and the lysis volume was measured (lower plot). For the assay of amidolytic activity with substrate S-2444, 2.8 n*M* plasmin was added to 60 μl aliquots. The reaction was stopped by addition of 200 kIU aprotinin/ml (upper plot). Amidolytic or fibrinolytic activity generated by 50 units of pro-uPA was defined as 100%.

amount of enzymatically active elastase was released (Table I). The addition of pro-uPA to supernatant obtained from stimulated granulocytes prevented the conversion of pro-uPA into enzymatically active uPA compared to supernatant obtained from unstimulated cells (Fig. 3). Supernatant of CHO-NLPNTL-stimulated granulocytes was incubated with different concentrations of either eglin C (elastase inhibitor) (0–12 μM) or anti-elastase moAB (blocks activity) (0–15 μM). Figures 4 and 5 show the effect of cell supernatant obtained from stimulated granulocytes on the amidolytic and fibrinolytic activity of pro-uPA in the presence of various amounts of eglin C or moAB with respect to elastase. In the presence of 12 μM eglin C, 96% of the latent enzymatic activity of pro-uPA was preserved (Fig. 4). With 15 μM moAB to elastase, 80% of the latent enzymatic activity was retained (Fig. 5).

Effect of granulocyte supernatant on fibrinolytic activity of cancer cells Fibrinolytic activity of A431 and MCF7 cells following various treatments is shown in Fig. 6. A431 and MCF7 cells in PBS buffer dissolved 0.30 ± 0.05 ml and 0.25 ± 0.03 ml, respectively. A431 and MCF7 cells treated with non-activated granulocyte supernatant also lysed 0.30 ± 0.04 and 0.27 ± 0.02, respectively. On the other hand, the lysis volumes of A431 and MCF7 cells incubated with activated granulocyte supernatant were 0.04 ± 0.03 and 0.01 ± 0.02, respectively. Thus, there is a significant difference between the fibrinolytic activity of the cells treated with activated granulocyte supernatant and the others. The inhibition rates of fibrinolytic activity of A431 and MCF7 cells by activated supernatant were 87% and 96%, respectively. Trypan blue staining gave nearly the same value for living cells

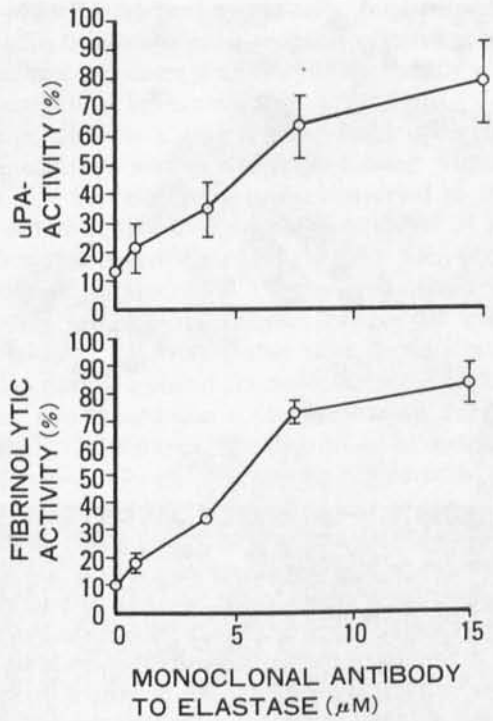


Fig. 5. Inhibition of elastase activity in granulocyte supernatant by moAB to elastase. Supernatant (60 μl) from granulocytes stimulated with 10^{-8} *M* CHO-NLPNTL in the presence of cytochalasin B (for details see "Materials and Methods" section) were incubated with different concentrations of moAB to elastase (0–15 μM) and then mixed with 50 units of pro-uPA in 60 μl of PBS (30 min, 37°C). Half the volume of the reaction mixture was applied to fibrin clots in tubes (2 h, 37°C) and the lysis volume was measured (lower plot). For the assay of amidolytic activity with substrate S-2444, 2.8 n*M* plasmin was added to 60 μl aliquots. The reaction was stopped by addition of 200 kIU aprotinin/ml (upper plot). Amidolytic or fibrinolytic activity generated by 50 units of pro-uPA was defined as 100%.

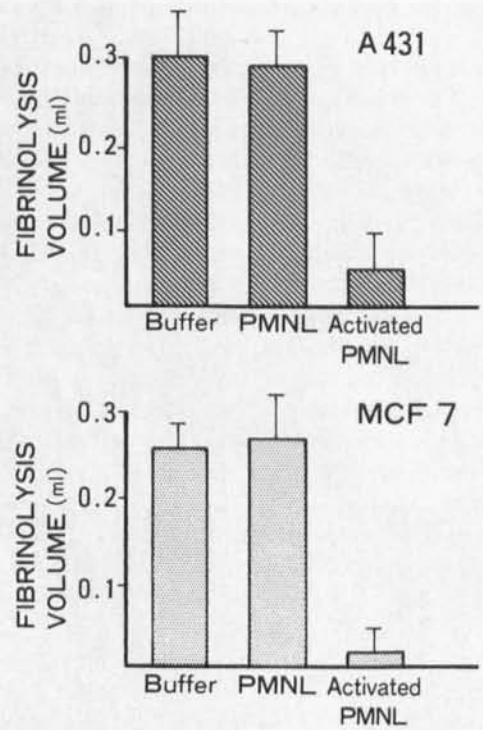


Fig. 6. Effect of granulocyte supernatant on fibrinolytic activity of cancer cells. Epidermoid cancer cells A431 (upper plot) or breast cancer cells MCF7 (lower plot) were incubated with supernatant (40 μl) from granulocytes or tris buffer (30 min, 37°C) containing sodium azide (0.25 m*M*). Aliquots (60 μl) were applied to a fibrin clot (12 h, 37°C). PMNL: Polymorphonuclear leukocytes.

Table II. Detection of uPA in Cancer Cell Lines

Cell line	uPA activity ($U/10^5$ cells)	uPA antigen ($ng/10^5$ cells)
A431	0.32 ± 0.07	3.1 ± 0.4
MCF7	0.27 ± 0.04	2.8 ± 0.2

After treatment with 0.1% Triton X for A431 and MCF7 (2×10^6 cells), uPA activity and antigen were measured by ELISA as described above.

(95% each) with or without granolocyte supernatant after preincubation. uPA activity and content of the cell lines are shown in Table II. The activity and content of A431 were similar to those of MCF7.

Localization of elastase and uPA in cancer tissues Cells containing uPA or elastase were localized in human uterine cervical and endometrial cancer tissues by moAB to the antigens. uPA was localized in the cytoplasm of the cancer cells in formaldehyde-fixed tissues of adenocarcinoma and squamous cell carcinoma (Fig. 7A, 7C). Cancer cells stained homogeneously with the uPA antibody, although differences in staining intensity within the tumors were observed.

Elastase-containing cells were localized in the tumor tissue and also the tumor stroma surrounding the tumor nests (Fig. 7B, 7D). These cells most probably represent granulocytes. The staining pattern of some of these phagocytic cells was irregular, indicating release of elastase into the tumor stroma and tumor cells.

DISCUSSION

Proteins in plasma and tissues, such as fibrin(ogen), fibronectin, elastin and collagen, have been shown to be substrates for elastase.[15,16] In this report we demonstrate

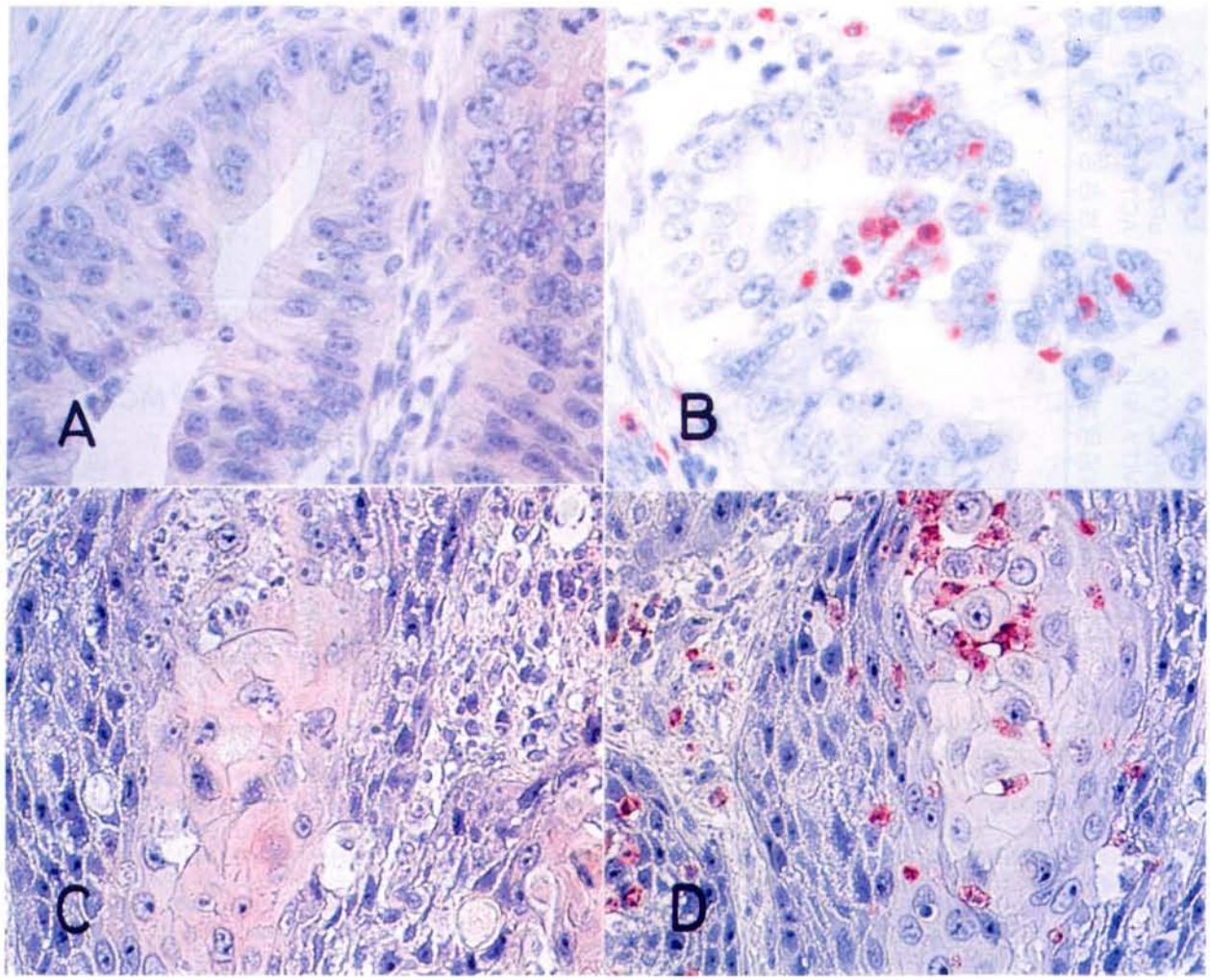


Fig. 7. Localization of elastase and uPA in uterine endometrial and cervical cancer tissues. Moderately differentiated endometrial (A, B) and cervical (C, D) carcinoma of a stage I patient. Areas selected represent the central part of the tumor. Immunohistochemical staining (pinkish color) was carried out for the presence of uPA in tumor cells (A, C) or elastase in phagocytes (B, D). The elastase-containing cells were located in the tumor stroma and also close to tumor cells. Nuclei (blue) stained with hematoxylin. Microscopic magnification: ×400.

that cancer tissues contain granulocytes which stain for elastase and tumor cells which stain for uPA. In *in vitro* experiments we have shown that purified tumor cell pro-uPA is degraded and inactivated by granulocyte elastase. Dose-dependent inactivation of pro-uPA by elastase was observed for amidolytic and fibrinolytic activity. Treatment of pro-uPA with 100 n*M* elastase completely prevented the generation of enzymatically active uPA by subsequent plasmin addition to the elastase-treated molecule. However, the inhibitory curves of elastase on uPA activity and fibrinolytic activity were different. This may be explained by the difference of active sites of the enzyme (uPA) in each reaction.

Treatment of pro-uPA with supernatants of chemotactically activated granulocytes markedly depressed the latent enzymatic capacity of pro-uPA *in vitro*. By inhibition experiments with eglin C or an antibody to the active site of elastase, elastase was identified as the key enzyme for this proteolytic activity. Heiple and Ossowski also reported the destructive potential of cell supernatants of stimulated granulocytes on pro-uPA without identifying the proteases involved.[17] The inactivation capacity of the cell supernatants was inhibited by the serine protease inhibitor diisopropylfluorophosphate (DFP). Elastase is inactivated by DFP. Heiple's and our results support the notion that elastase is one of the key enzymes in inacti-

vating pro-uPA released by the cells. *In vivo* inactivation of pro-uPA by elastase most probably occurs in the extravascular space, because granulocytes are usually activated after penetrating from vessels.

Cancer tissue is a case where phagocytic cells such as granulocytes and pro-uPA-containing tumor cells coexist.[18-22] Secreted pro-uPA is converted to enzymatically active HMW-uPA by small amounts of plasmin and then tumor-associated fibrinolysis can occur.[23,24] Degradation products of the tumor stroma (fibrin-fibronectin matrix) may attract phagocytic cells into tumor stroma.[25] Recent studies have demonstrated that even uPA itself is a potent chemotactic factor for granulocytes *in vivo* in addition to tumor stroma degradation products.[26,27] However, the functions of granulocytes around tumor cells are little known compared with those of macrophages and lymphocytes. One of the functions attributed to granulocytes accumulated around tumor cells is a cytotoxic effect on the tumor by release of reactive oxidative intermediates upon stimulation. These oxidants are supposed to destroy tumor cells directly.[28,29] Now, we have found that activated granulocytes depress invasion of cultured cancer cells into a fibrin matrix. Under our experimental conditions, since we added sodium azide to the reaction mixture, the influence of radical oxygen on the cancer cells could be neglected.[30] Therefore, inhibition of fibrinolytic activity of cancer cells would be mainly due to elastase released from granulocytes.

The immunohistochemical study also supported this hypothesis. Immunohistochemical staining of cancer sections demonstrated that elastase is localized in cells close to uPA-rich cancer cells and in the tumor stroma surrounding the tumor nests. In those regions pro-uPA released by tumor cells could be inactivated by elastase released from granulocytes. Dvorak *et al.* observed that granulocytes can attach to tumor cells and then elastase-containing granules may be released.[31] The concentration of protease inhibitors in such regions should be lower than in plasma and would not be sufficient to neutralize elastase activity. These findings lead one to speculate that pro-uPA of tumor cells could be inactivated effectively by elastase in tumor tissue. This may have important implications in tumor biology. Inactivation of pro-uPA by elastase may prevent the generation of enzymatically active uPA and thus diminish tumor cell metastasis and invasion.

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