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Activation of pro-urokinase by the human T cell-associated serine proteinase HuTSP-1

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The human T cell-associated serine proteinase-1 (HuTSP-1) is expressed by activated T lymphocytes and is exocytosed upon their interaction with target cells. Here, we report that HuTSP-1 is able to convert single-chain human pro-urokinase into the active two-chain enzyme. Time-dependent activation by HuTSP-1 of recombinant human pro-urokinase as well as natural pro-urokinase derived from human melanoma cells was demonstrated in a chromogenic assay specific for active urokinase type plasminogen activator and in immunoblotting experiments revealing the conversion of single-chain into two-chain urokinase. Control experiments excluded plasmin as the activating agent. These data suggest a novel pathway for plasmin generation during T cell-mediated processes such as immune responses and extravasation of immune cells.

Plasminogen activator; Urokinase; Pro-urokinase; Proenzyme activation; T cell-associated serine proteinase-1

1. INTRODUCTION

PAs catalyze the proteolytic activation of the proenzyme plasminogen to plasmin, a trypsin-like proteinase of broad specificity. The substrate for PAs, plasminogen, is synthesized in the liver and is present in all body fluids constituting a source for the generation of extracellular proteolysis via cellular secretion of PAs (for a review, see [1]). Two types of human PAs have been identified, which are produced by a variety of cells: uPA and tPA. Although both enzymes are evolutionarily related, they are immunologically distinct and are encoded by two independent genes [2,3]. Moreover, they seem to have different biological functions, as tPA is mainly responsible for fibrin clot lysis, whereas uPA was found to be associated with processes involving cell migration and tissue degradation including inflammatory reactions [4] as well as tumor cell invasion [5,6] and metastasis [7,8]. In general, cells secrete uPA as an inactive single-chain proenzyme (pro-uPA) [9,10] exhibiting only 4–6% of the plasminogen activating activity of two-chain uPA [11]. Efficient plasminogen activation requires the conversion of pro-uPA into active uPA by limited proteolysis, that can be exerted by plasmin [12], plasma kallikrein, or trypsin [13]. Moreover, it is assumed that as yet unidentified cellular proteinases are able to catalyze pro-uPA activation as well [15]. On the other hand, it has been shown that pro-uPA is proteolytically inactivated by thrombin or leukocyte elastase [13,14].

Human CD 4^+ and CD 8^+ T lymphocyte lines express a disulfide-linked homodimeric serine proteinase termed HuTSP-1 [16]. HuTSP-1 (molecular mass, 50 kDa) is expressed in activated, but not in resting T lymphocytes in vitro and in vivo [16,17]. The enzyme is confined to cytoplasmic storage granules, the contents of which are released into the extracellular space upon antigen binding to the T3/Ti T cell receptor complex. By using defined peptide substrates, HuTSP-1 was found to have trypsin-like specificity [16,18].

Here, we report that HuTSP-1 is able to catalyze the conversion of human pro-uPA into active uPA. Thus, T cell-associated serine proteinases are candidates to trigger the enzymatic cascade of plasminogen activation, which could be relevant during the generation of immune reactions as well as for the invasiveness of activated or malignant T lymphocytes.

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Abbreviations: PA, plasminogen activator; pro-uPA, pro-urokinase; rpro-uPA, recombinant pro-urokinase; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; tPA, tissue type plasminogen activator; uPA, urokinase type plasminogen activator; HuTSP-1, human T cell-associated serine proteinase-1

2. MATERIALS AND METHODS

Rpro-uPA (231 260 IU/mg; more than 93% pure, containing less than 0.4% active uPA) was a generous gift from Grünenthal (Stolberg, FRG). Human plasmin (P 4895, 3.3 units/mg) and aprotinin were obtained from Sigma (St. Louis, USA), the chromogenic substrates S-2444 (Glu-Gly-Arg-NH-*p*-nitroanilide × HCl), S-2251 (H-D-Val-Leu-Lys-NH-*p*-nitroanilide × 2 HCl), and

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S-2288 (H-D-Ile-Pro-Arg-NH-*p*-nitroanilide × 2 HCl) from Kabi (Mölndal, Sweden). Rabbit anti-human plasminogen IgG was purchased from Behring (Marburg, FRG) and goat anti-rabbit IgG conjugated with alkaline phosphatase from Dianova (Hamburg, FRG).

The human melanoma cell line Mel-Juso [19] secreting uPA was cultured in RPMI 1640 supplemented with 10% fetal calf serum in a humid atmosphere in 5% CO_2. Conditioned medium containing pro-uPA was prepared by seeding the cells at a density of 2×10^5 cells/ml in RPMI one day before adding the serum-free HL-1 medium (Paesel and Lorei, Frankfurt, FRG). One day thereafter the supernatants were harvested containing uPA (95% pro-uPA, 5% active uPA), the total activation of which resulted in 30–50 IU/ml uPA activity.

HuTSP-1 was purified from the Cd 8^+ T lymphocyte clone UA B34.C7. The enzyme content of these cells is about 70 units HuTSP-1/10^6 cells [16]. One unit is defined as the amount of enzyme producing a ΔE_{405} of 0.01/h incubation at 37°C using the chromogenic substrate S-2288. Purification of HuTSP-1 was performed as previously described [18]. Briefly, cells were lysed at a cell density of 5×10^7/ml in 10 mM Tris-HCl (pH 7.5) containing 0.1% (v/v) Triton X-100. After 1 h on ice, lysates were centrifuged at 3000 × *g* to remove particulate material, adjusted to 1 mg/ml heparin and applied to a *p*-aminobenzamidine Sepharose column (Pharmacia, Uppsala, Sweden), equilibrated with 0.1 M Tris-HCl (pH 8.0) containing 1 mg/ml heparin (buffer A). Irrelevant protein was removed from the column with a linear salt gradient ranging from 0 to 1 M sodium chloride in buffer A. HuTSP-1 was eluted with 1 M arginine-HCl in buffer A. By SDS-PAGE under non-reducing or reducing conditions and silver staining, the eluate appeared as a single protein band incorporating [^{3}H]diisopropyl fluorophosphate and migrating according to a molecular mass of 50000 or 25000, respectively. Amino acid analysis further demonstrated that HuTSP-1 is a homodimeric molecule [18]. The specific activity of this enzyme preparation was 14100 units/mg protein.

Pro-uPA in Mel-Juso culture supernatant (30–50 IU/ml HL-1 medium) or rpro-uPA (100 IU/ml 50 mM Tris-HCl, pH 8.5, containing 38 mM sodium chloride) were activated by addition of HuTSP-1 or plasmin followed by incubation at 37°C. The reaction was stopped by addition of aprotinin (100 KIU/ml) and then active uPA was quantified with the chromogenic substrate S-2444 [20].

Plasmin activity in aliquots of 60 μl was determined by addition of 20 μl of 30 mM Tris-HCl, pH 7.4, containing 60 mM sodium chloride and 20 μl of the plasmin substrate S-2251 (2 mg/ml in the same buffer).

The ELISA for plasminogen/plasmin was performed using polyclonal rabbit anti-plasminogen IgG as primary ('catching') antibody and a monoclonal antibody specific for plasminogen/plasmin as detecting antibody [21].

3. RESULTS

Pro-uPA or rpro-uPA were incubated with HuTSP-1. Activation of pro-uPA and rpro-uPA was tested in a chromogenic assay using the uPA-specific substrate S-2444, which was not cleaved significantly by pro-uPA or HuTSP-1. Fig.1A shows time-dependent generation of active uPA by HuTSP-1 in both pro-uPA preparations. After 24 h 29.6 IU/ml rpro-uPA or 56% of the melanoma cell-derived pro-uPA, respectively, were activated by 1 unit/ml HuTSP-1. The amount of HuTSP-1 used for the experiment (1 unit) is secreted by about 3×10^5 activated UA B34.C7 cells per day [16].

The HuTSP-1-catalyzed conversion of single-chain rpro-uPA into active two-chain uPA was also demonstrated in immunoblotting experiments revealing

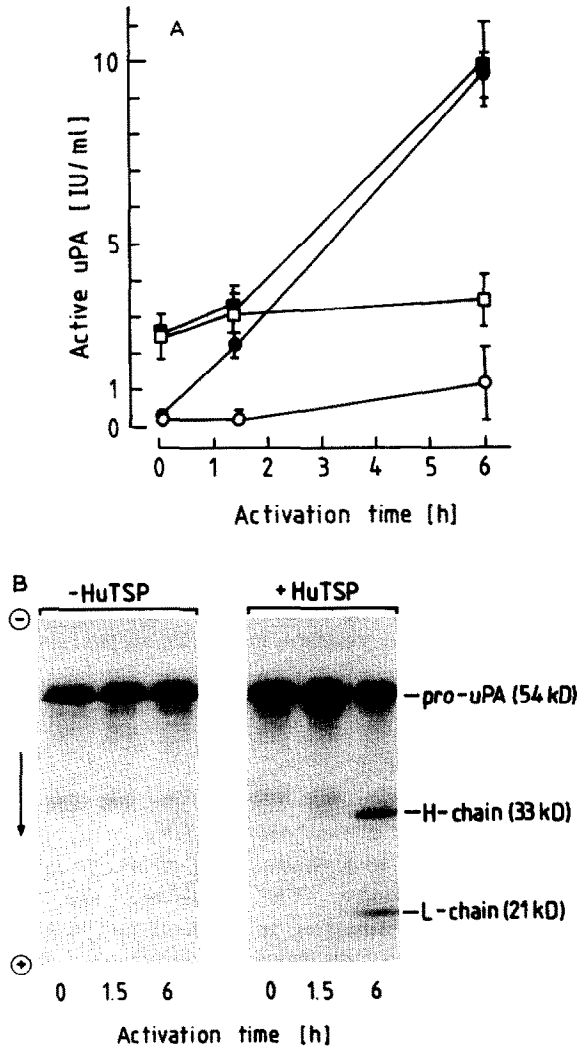

Fig.1. Pro-uPA activation by HuTSP. (A) Melanoma-derived human pro-uPA (30–50 IU/ml Mel-Juso culture supernatant) (□, ■) or Pro-uPA (100 IU/ml) (○,●) were incubated at 37°C with (filled symbols) or without (open symbols) HuTSP-1 (1 unit/ml) and the generated active uPA was quantified using the chromogenic substrate S-2444. (B) Rpro-uPA (100 IU/ml) was incubated at 37°C with HuTSP (1 unit/ml). Aliquots of 100 μl containing about 43 ng rpro-uPA and 7 ng HuTSP-1 were applied to SDS-PAGE under reducing conditions [23] in 7.5–15% polyacrylamide gradients followed by the electrophoretic transfer of the proteins onto nitrocellulose filters. Protein bands related to uPA were visualized by immunoblotting using rabbit anti-human uPA IgG (10 μg/ml) and goat anti-rabbit IgG coupled to alkaline phosphatase (1:10000).

the appearance of the heavy and light chain of active uPA (fig.1B).

In order to exclude a contamination of the HuTSP-1 preparation with trace amounts of plasmin, a known activating agent of pro-uPA, we determined the amount of plasmin required to convert a proportion of pro-uPA into active uPA similar to that activated by the HuTSP-1 preparation. At a concentration of ≈2×10^{-4} units/ml, plasmin (0.7 nM) was found to be functionally equivalent to the HuTSP-1 preparation

Table 1

Demonstration of the absence of plasmin in the HuTSP preparation

	Pro-uPA activation (%/h)[a]	Plasmin activity (ΔE_{405}/24 h)[b]	Plasmin antigen (ΔE_{482}/5 min)[c]
HuTSP (1.4 units/ml)	4.1	n.d.[d]	n.d.
Plasmin (10^{-4} units/ml)	2.2	0.020	0.165
(3×10^{-4} units/ml)	5.9	0.092	0.526

[a] The generation of active uPA in Mel-Juso culture supernatant was quantified by incubation at 37°C with the chromogenic substrate S-2444 and expressed as percent of total activatable pro-uPA
[b] Determined by incubation at 37°C with the chromogenic substrate S-2251
[c] Determined in an ELISA assay for plasmin
[d] Not detectable

(1.7 nM) and was easily detectable either in a chromogenic assay using the plasmin substrate S-2251 or in an ELISA assay using antibodies specific for plasmin (table 1). On the other hand, no plasmin could be detected in the HuTSP-1 preparation using either assay system, demonstrating that the activation of pro-uPA by the HuTSP-1 preparation is not catalyzed by contaminating plasmin.

4. DISCUSSION

Initiation of the proteolytic cascade of plasminogen activation requires enzymatically active PAs, which are generated from its inactive single-chain proenzyme by limited proteolysis [9,10]. Up to now, humoral serine proteinases such as plasmin or kallikrein have been found to catalyze pro-uPA activation [12,13]. Here, we demonstrated that HuTSP-1, a serine proteinase associated with T effector cells of both subsets CD 4^+ and CD 8^+ [16], is also able to process natural as well as recombinant human pro-uPA to yield the enzymatically active two-chain uPA. On a molar basis the efficiency of the activation process catalyzed by HuTSP-1 or plasmin is in the same order of magnitude (table 1). Contaminating plasmin as the activating principle in the HuTSP-1 preparation was excluded.

HuTSP-1 is selectively expressed in activated T cells. The enzyme, which has also been demonstrated in inflammatory foci in vivo [17], is contained within intracellular storage granules and secreted by vectorially oriented exocytosis upon interaction of T effector cells with appropriate target cells [16]. Under pathophysiological conditions, secreted HuTSP-1 may thus induce the generation of both uPA and plasmin by activating pro-uPA, which has been found in a variety of tissues and cells, including those participating in inflammatory reactions such as macrophages [4]. In addition to processing plasminogen, active uPA is also mitogenic for T lymphocytes [22], suggesting that activation of pro-uPA via HuTSP-1 might contribute to the process of chronic inflammation by perpetuating destruction of extracellular tissue components as well as cellular proliferation.

Recently, the extracellular prevalence of two-chain uPA has been demonstrated in leukemic cells of various lymphoid phenotype [15]. On the other hand, inactive single-chain uPA was predominant in tumor cells derived from solid tumors. On the basis of the results reported here, it could be speculated that T cell leukemias may activate newly synthesized pro-uPA by endogenously produced and secreted HuTSP-1, thereby increasing their invasive potential. Future studies will focus on the putative coexpression of HuTSP-1 and pro-uPA by malignant T lymphocytes and their role in extracellular proteolysis during invasion and metastasis.

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