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Longistatin, a novel plasminogen activator from vector ticks, is resistant to plasminogen activator inhibitor-1

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ABSTRACT

Thrombo-occlusive diseases are major causes of morbidity and mortality, and tissue-type plasminogen activator (t-PA) is recommended for the treatment of the maladies. However, both t-PA and u-PA are rapidly inactivated by plasminogen activator inhibitor-1 (PAI-1). Here, we show that longistatin, a novel plasminogen activator isolated from the ixodid tick, *Haemaphysalis longicornis* is resistant to PAI-1. Longistatin was relatively less susceptible to the inhibitory effect of SDS-treated platelet lysate than physiologic PAs. Platelet lysate inhibited t-PA and tcu-PA with the IC_{50} of 7.7 and 9.1 μg/ml, respectively, whereas for longistatin inhibition IC_{50} was 20.1 μg/ml ($p < 0.01$). Similarly, activated PAI-1 (20 nM) inhibited only 21.47% activity of longistatin but almost completely inhibited t-PA (99.17%) and tcu-PA (96.84%). Interestingly, longistatin retained 76.73% initial activity even after 3 h of incubation with 20 nM of PAI-1. IC_{50} of PAI-1 during longistatin inhibition was 88.3 nM while it was 3.9 and 3.2 nM in t-PA and tcu-PA inhibition, respectively. Longistatin completely hydrolyzed fibrin clot by activating plasminogen efficiently in the presence of 20 nM of PAI-1. Importantly, unlike t-PA, longistatin did not form complex with PAI-1. Collectively, our results suggest that longistatin is resistant to PAI-1 and maybe an interesting tool for the development of a PAI-1 resistant effective thrombolytic agent.

1. Introduction

Thrombo-occlusive events including myocardial infarction, ischemic stroke, obstructive pulmonary disease and peripheral thromboembolism are major causes of morbidity and mortality in humans. Most countries are facing a high and increasing rate of cardiovascular diseases. In each year, heart diseases kill more Americans than does cancer. In women, death due to cardiovascular diseases is higher than that caused by breast cancer [1–4]. Treatment of thrombo-occlusive diseases is one of the most challenging areas in medicine. Plasminogen activators (PAs) have great clinical significance as thrombolytic agents in the managements of cerebral and cardiac attacks [5]. PAs such as tissue-type plasminogen activator (t-PA), urokinase-type plasminogen activator (u-PA) and others activate plasminogen, a circulating plasma zymogen, into its active form plasmin, a powerful serine protease, which in turn dissolves thrombi and restores circulation in thrombosed vasculature; thus, rescue the affected tissues/organs from the devitalizing events of thrombosis [1,6–8]. Among the PAs recommended for clinical use, t-PA is generally preferred for its relatively higher efficacy and wider safety window compared to u-PA and streptokinase. However, both t-PA and u-PA are rapidly inactivated by the fast-acting serine protease inhibitor (serpin) plasminogen activator inhibitor-1 (PAI-1), a molecular switch of physiologic PAs, and is the major PAI in the serum [9,10]. PAI-1 plays crucial roles in the regulation of PA mediated thrombolysis [1,6]. PAI-1 is synthesized and secreted by a variety of cells such as endothelium, adipocytes, monocytes/macrophages, fibroblasts, cardiomyocytes, hepatocytes, smooth muscle cells, granulosa cells and megakaryocytes, and stored in the α-granules of platelets, and is released following activation of platelets [10,11]. PAI-1 forms a tight complex with PAs and rapidly neutralizes them. In fact, exogenous PAs have to work after saturating the PAI-1 and other serpins present in serum

Abbreviations: t-PA, tissue-type plasminogen activator; u-PA, urokinase-type plasminogen activator; tcu-PA, two chain u-PA; serpin, serine protease inhibitor; PAI-1, plasminogen activator inhibitor-1; SDS–PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; PRP, platelet-rich plasma; PPP, platelet-poor plasma.

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that hinder thrombolytic therapy to some extent [6,12]. Therefore, development of PAI-1 resistant PA is essential for the betterment of effective thrombolytic therapy.

Recently, we have isolated and identified longistatin, a novel PA from salivary glands of the ixodid tick, *Haemaphysalis longicornis*, a common vector of many deadly diseases of humans and animals [13,14]. Longistatin is a 17.8-kDa secretory protein with an N-terminal sequence of unknown structure and two calmodulin-like functional EF-hand domains at the C-terminus. Longistatin is synthesized in, and secreted from the salivary glands with saliva and injected into host tissues during acquisition of blood-meal [15]. Longistatin, like physiologic PAs can efficiently activate plasminogen and cause lysis of fibrin clot and fresh thrombi; thus, plays crucial roles in the development and maintenance of the blood pool, the essential feeding lesion of hard ticks, throughout the entire feeding period keeping blood in a fluid state. Longistatin binds with fibrin meshwork with higher affinity than t-PA, and fibrin acts as co-factor during plasminogen activation processes [16]. Here, we show that longistatin is relatively resistant to SDS-treated platelet lysate. Longistatin is refractory to the inhibitory effects of activated, purified and commercially available PAI-1. Longistatin sufficiently activated plasminogen into plasmin in the presence of PAI-1 (20 nM), and completely hydrolyzed fibrin clot. Furthermore, longistatin does not form any complex with PAI-1. To our knowledge, longistatin is the first PA identified and characterized from arthropods.

2. Materials and methods

2.1. Production of recombinant longistatin

Recombinant longistatin was produced and purified as described previously [15]. Protein was concentrated using Centrisart® (Sartorius) having a mol. wt cut-off of 10 kDa. His-tag was removed from longistatin by incubating with enterokinase (EKMax™, Invitrogen) and purified using the enterokinase-eliminating column (Ek-Away™ Resin, Invitrogen) following the manufacturer's protocol. The concentrated protein was dialyzed extensively at 4 °C with successive changes of 20 mM Tris–HCl (pH 7) and a decreasing concentration of NaCl (500–250 mM) using a Slide-A-Lyser Dialysis Cassette (Pierce) with a mol. wt cut-off of 10 kDa. N-terminal and internal amino acid sequences of purified longistatin were confirmed by automated Edman's degradation. Purity of the recombinant was judged employing one and two dimensional electrophoresis followed by silver staining technique. The concentration of longistatin was determined using micro-BCA reagents (Pierce) as described previously [15].

2.2. Blood collection and preparation of plasma and serum

Blood samples were collected from fully consented healthy volunteers into EDTA-treated tubes. Platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were produced following the procedures as described [6,17] with slight modifications. Briefly, PRP was produced by centrifuging blood at 100*g* and 23 °C for 10 min, and supernatant was collected. To prepare PPP, PRP was chilled at 4 °C for 15 min and centrifuged at 2000*g* and 4 °C for 30 min, and supernatant was carefully collected without disturbing the pellet. To prepare serum, 1 ml of either whole blood, PRP or PPP was treated with 5 μl of thrombin (0.1 NIH unit/μl, Sigma) and incubated at 37 °C for 1 h. The clot was centrifuged at 10,000*g* and 4 °C for 30 min and supernatant was collected. Plasma and serum were stored at −20 °C until further use.

2.3. Preparation of platelet lysate

PRP (1 ml) from human blood was chilled at 4 °C for 15 min and centrifuged at 2000*g* and 4 °C for 30 min; then, PPP was aspirated. The pellet was extensively washed with PBS. The platelets were incubated with thrombin (0.5 U/ml, final concentration) at 37 °C for 6 h in Tyrode's buffer (pH 6.5). The platelets were sonicated with an ultrasonic processor (VP-5S, TAITEC) for 2 min on ice with three pauses each of 25 s, and centrifuged at 22,300*g* and 4 °C for 1 h. Supernatant was collected and treated with 0.1% SDS at 37 °C for 1 h; then, SDS was neutralized treating with 1% tritonX-100 at 4 °C for 1 h as described previously [17]. SDS-treated platelet lysate was stored at −20 °C until further use.

2.4. Treatment of longistatin with platelet lysate

An equal amount (1 μg) of longistatin, t-PA (Calbiochem) or tcu-PA (CosmoBio Co. LTD) was reacted with various amount of SDS-treated platelet lysate (0–30 μg/ml) in a total volume of 25 μl of buffer A (50 mM Tris–HCl, pH 7; 100 mM NaCl and 5 mM $CaCl_2$) at 37 °C for 15 min. Then, the mixture was immediately diluted with buffer A. Plasminogen (5 μl, 0.2 μg/μl, Sigma) was added in the reaction mixtures and incubated at 37 °C for further 1 h in the absence (incase of tcu-PA) or presence (incase of t-PA/longistatin) of soluble fibrin (8 μg/ml, Technoclone). Residual activity of plasminogen activator was determined employing indirect fluorogenic assays using a plasmin-specific synthetic substrate (Boc-2Glu-Lys-Lys-MCA, Peptide Institute). Substrate hydrolysis was monitored by measuring excitation and emission wavelengths of 360 nm and 460 nm, respectively, using a Spectra Fluor fluorometer (TECAN). Percentage of inhibition of the enzyme was calculated using the following formula:% inhibition = (1 − inhibited rate/uninhibited rate) × 100 and IC_{50} was determined as described previously [18].

2.5. Inhibition of longistatin with PAI-1

An equal amount (1 μg) of longistatin, t-PA or u-PA was incubated with commercially available, activated PAI-1 (ITSI Bioscience) at various concentrations (0–90 nM) at 37 °C for different time (0–180 min) in a total volume of 25 μl of buffer A. Then the reaction mixture was immediately diluted adding buffer A and residual activity was determined using a synthetic substrate specific for t-PA/u-PA (Pyr-Gly-Arg-MCA, Peptide Institute). Substrate hydrolysis was monitored by measuring excitation and emission wavelengths of 360 nm and 460 nm, respectively, using a Spectra Fluor fluorometer (TECAN). Percentage of inhibition of enzyme and IC_{50} were determined as described above.

2.6. Microplate clot lysis assay

We produced fibrin clot in a 96-well plate by incubating 3 μl of fibrinogen (7.5 mM in final concentration, Sigma) and 2 μl of thrombin (0.10 NIH unit/μl, Sigma) in a total volume of 100 μl of buffer A at 37 °C for 1 h. An equal amount (1 μg) of t-PA, tcu-PA or longistatin was mixed with PAI-1 (20 nM, in final concentration) in a total volume of 90 μl of buffer A and incubated for 15 min at 37 °C. Then, plasminogen (10 μl, 0.2 μg/μl, Sigma) was mixed with each reaction mixture. The mixtures were gently added to the fibrin clot separately and incubated at 37 °C for 6 h. Only buffer A was added as a negative control and longistatin alone was used as a positive control. Lysis of fibrin clot was detected visually and also by measuring changes in turbidity at 450 nm using a spectrophotometer (TECAN) up to 6 h.