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Role of isopeptidolysis in the process of thrombolysis

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ABSTRACT

Introduction: Known thrombolytic agents either break peptide bonds in the fibrin molecule or act as plasminogen activators, which also results in peptide bond cleavage. In thrombi, fibrin molecules are known to be cross-linked by isopeptide bonds, the formation of which is mediated by factor XIIIa. In this work, we studied the dissolution of thrombi via isopeptide bond cleavage using a recombinant destabilase. Destabilase is an enzyme secreted from the medicinal leech salivary gland. This enzyme exhibits muramidase (lysozyme) activity, in addition to *endo*-ε-(γ-Glu)–Lys-isopeptidase activity, which is responsible for isopeptide bond cleavage.

Methods: Venous (jugular vein) and arterial (carotid artery) thrombosis was induced in rats. Rats were intravenously injected with both recombinant destabilase produced in *Escherichia coli* and a commercial streptokinase preparation. After 24 h, the weight and degree of cross-linking in the thrombi were analysed. Amidolytic activity in rat blood serum was measured in order to evaluate destabilase levels in the blood.

Results: Destabilase was definitively shown to cause a 47.6% and 74.6% decrease in the weight of venous and arterial thrombi, respectively. The enzyme proved to be more efficient at dissolving thrombi compared to streptokinase. The combined administration of destabilase and streptokinase has a greater effect than the injection of individual enzymes. Destabilase reduces fibrin stabilization in thrombi.

Conclusion: Cumulatively, we find that the medicinal leech destabilase is a more efficient thrombolytic agent for dissolving thrombi, which could help increase the overall effectiveness of conventional thrombolytic drugs.

1. Introduction

The principal goal of thrombolytic therapy is to destroy or dissolve a thrombus that forms in the body as a result of thrombophilia [1]. Therefore, the search for and design of effective thrombolytic medicines is a pressing issue. Thrombolytic drugs currently in clinical use consist of various modified serine proteases involved in the fibrinolytic system of the blood [2], bacterial and plant proteases [3,4], and metalloproteinases [5]. Their mechanisms of action involve the proteolytic degradation of polypeptide bonds in fibrin polymers, which are a core component of a thrombus. In this work, we describe a fundamentally new mechanism of thrombolysis called "thrombolysis-isopeptidolysis". This process is activated by destabilase, an enzyme secreted from the medicinal leech salivary gland, which was first described by our group in 1985 [6]. After the discovery of destabilase and related proteins, a new conserved domain (pfam05497:Destabilase) was added to NCBI's conserved domain database (https://www.ncbi.nlm.nih.gov/cdd). Destabilase is an i-type lysozyme. Apart from exhibiting muramidase activity, this enzyme catalyses the hydrolysis of *endo*-ε-(γ-Glu)-Lys-isopeptide bonds or cross-links that form between the γ-γ chains and α-α chains of fibrin monomers in thrombi, which are catalysed by blood plasma transglutaminase (factor XIIIa) [7]. It is known that more extensively cross-linked thrombi are less sensitive to degradation. This is why thrombi are difficult to manage [8,9].

We used recombinant destabilase to evaluate the degree of fibrin stabilization in arterial and venous thrombi. Destabilase effectively dissolved arterial and venous thrombi in experimental animals. This effect was achieved without the activation of a thrombolytic agent (such as protease plasmin) or the presence of a plasmin activator (streptokinase).

2. Materials and methods

2.1. Animals

Sprague Dawley (SD) rats weighing 450–550 g were obtained from the Nursery for Laboratory Animals "Pushchino". After a 14-day quarantine, clinically healthy animals were ranked by weight and distributed into experimental groups.

The rats were housed in the vivarium of the Faculty of Biology at

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Lomonosov Moscow State University. The room temperature was maintained at 20–24°C at a relative humidity of 50 ± 20%. The light cycle was 12-h light/12-h dark. Animals received the extruded complete feed “Chara” (JSC “Assortiment-Agro”) and filtered autoclaved tap water ad libitum. The cages were cleaned and bedding was changed at least once per week. A total of 62 animals were used. Care and use of the laboratory animals involved in this research was carried out in accordance with EU Directive 2010/63/EU for animal experiments, FELASA recommendations (e.g. Guidelines for the veterinary care of laboratory animals (2008), Recommendations for health monitoring of rodent and rabbit colonies (2014)) and Lomonosov Moscow State University Commission on Biological Ethics recommendations on laboratory animals use in the experiment. All experimental procedures applied in the study were conducted in accordance with Standard Operating Procedures (SOPs) validated in the laboratory. Investigators were trained to perform manipulations properly to get the admission to operate with laboratory animals.

2.2. Experimental design

Animals were distributed into four experimental groups: a negative control group (22 animals), positive control group (14 animals), experimental thrombosis-1 group (14 animals) and experimental thrombosis-2 group (12 animals). Each group was divided into two equal subgroups: arterial and venous.

Thrombosis was induced in anesthetized rats by intramuscular injection of Rometar (xylazine) at a dose of 5.2 mg/kg and intraperitoneal injection of Zoletil at a dose of 25 mg/kg. Each animal's respiration and heart rate were visually monitored. After these procedures, the animals were returned to their home cages, and the recovery from anaesthesia was assessed. At 24 h after thrombosis induction, the animals in the negative control group were injected with 1 ml of physiological saline solution via the tail vein. The animals in the positive control group were injected with a commercial streptokinase preparation (0.37 mg streptokinase per 1 mg of the preparation; RUE Belmedpreparaty, Republic of Belarus), which was determined by mass spectrometry [10] to be a dosage equivalent to the 250,000 IU normally administered to humans. A sample containing 1.3 mg of active streptokinase was dissolved in 1 ml of water, and the solution was injected into rats via the tail vein in two portions (0.5 ml each) at 30-min intervals. The dosage of the active protein was 2.6 mg/kg. The animals of the experimental thrombosis-1 group were injected with recombinant destabilase at a dose of 0.4 mg/kg (the preparation and purification of the protein were described in a previous study [11]). The animals of the experimental thrombosis-2 group were injected with 1 ml of a mixture of streptokinase and destabilase. Immediately before the injection, streptokinase (1.3 mg of the active protein) was dissolved in a solution of destabilase at a concentration of 0.2 mg/kg. Therefore, the total dose of streptokinase for the animals of this group was 2.6 mg/kg of the active protein, and the dose of destabilase was 0.4 mg/kg. All preparations were injected intravenously via the tail vein.

To assess amidolytic activity in the plasma, blood was collected from all animals at three time points: 1 d before the injections and 1 h and 24 h after the injections. Blood samples were collected from anesthetized animals. Before the procedure, rat tail was disinfected with 70% ethanol. After the complete evaporation of the alcohol, a small piece of skin from the tip of the tail was cut with sterile surgical scissors. Blood drops from the tip of the tail were collected in a test-tube. To accelerate blood collection experimenter ran fingers along the lateral surfaces of the tail in caudal direction slightly pressing the tail. Blood was stopped with a new tampon with 70% ethanol after reaching the required sample volume (400–500 μl). The animals were anesthetized at 1 h and 24 h after the injection of the preparations, third portion of blood samples was taken, access to operated vessel was opened by separating tissues along pre-made surgical suture. The presence or absence of vessel recanalization was visually inspected. Thrombi were extracted to assess the degree of lysis and the degree of fibrin stabilization. Finally, the animals were euthanized without recovery from anaesthesia.

2.3. Model of venous thrombosis

Venous thrombosis was induced using a 10% $FeCl_3$ solution added to the surface of an isolated segment of the rat jugular vein [12] in accordance with the recommendations of 2014 [13]. A 15–20-mm segment of the jugular vein was isolated from anesthetized rats under aseptic conditions. Two ligatures were applied to the vessel, and a trap was placed to fix the position of the thrombus in the vessel. The lower ligature was tightened for 15 min, allowing the blood to accumulate in the vein, and then the upper ligature was tightened. Filter paper presoaked in a 10% $FeCl_3$ solution was placed on the surface of the vessel. The surrounding tissues were isolated using a polyethylene film layer. After 15 min, the filter paper was removed and the surgical field was thoroughly washed with saline, the ligatures were untightened and removed, and the “trap” was left on the vessel. The wound was then sutured and treated with antiseptics.

2.4. Model of arterial thrombosis

Arterial thrombosis was induced by 10% $FeCl_3$ added to the surface of an isolated segment of the rat carotid artery [14]. After separating a 15–20-mm segment of the carotid artery, a piece of sterile polyethylene film was placed under this segment. A glass rod 2 mm in diameter was placed under the cranial region of the vessel. Downstream of the glass rod, an isolated segment of the artery was covered with a piece of filter paper (5 × 1.5 mm) pre-soaked with 10% $FeCl_3$. Vessel occlusion and the formation of an obturating thrombus were assumed to have occurred when the blood flow through the artery above the glass rod ceased. After 15 min, the application was removed. The wound was washed three times with physiological saline, closed in a layer-by-layer fashion, and treated with antiseptics.

The conditions used to induce a carotid arterial thrombosis were similar to the methods used to induce venous thrombosis formation (e.g., injury to the blood vessel wall, disruption of the blood flow, and enhanced coagulability). It should be noted that the vascular spasm favouring thrombolytic events plays a key role in this process [15].

2.5. Assessment of amidolytic activity in rat blood serum

After blood was collected from the tail vein, the test tubes were incubated at room temperature for 30–40 min until it was completely clotted. The blood was then centrifuged at 1500*g* for 10 min. After centrifugation, the serum was transferred into new test tubes and frozen at −70°C. The amidolytic activity in blood serum was determined by spectrophotometry at 405 nm relative to the chromogenic substrate L-γ-Glu-pNA (Sigma, USA), which is a model analogue of the diisopeptide ε-(γ-Glu)-Lys, the natural substrate of destabilase. The mixture was preincubated for 20 h at 22°C [16].

2.6. Determination of thrombus weight

The thrombi were removed from the vessels and washed with physiological saline. Excess moisture was removed with a filter paper followed by drying to a constant weight at 60°C. Each thrombus was then weighed using an analytical balance to an accuracy of 0.1 mg.

2.7. Evaluation of fibrin stabilization in thrombi

The method used to assess fibrin stabilization was based on the ability of non-stabilized fibrin to dissolve in 2% acetic acid, which is not observed for fibrin stabilized by isopeptide cross-linking. Dried thrombi were placed in polypropylene test tubes, and 2% acetic acid (2 ml) was

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