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Full Length Article

Plasminogen activator inhibitor type 1 in platelets induces thrombogenicity by increasing thrombolysis resistance under shear stress in an in-vitro flow chamber model

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ABSTRACT

Introduction: Despite the proven benefits of thrombolytic therapy with tissue plasminogen activator (t-PA) for peripheral thromboembolism, perfusion failure frequently occurs, particularly in arterial circulation. We evaluated how the modification of fibrinolytic activity affects thrombus formation under flow and static conditions.
Materials and methods: t-PA-treated human whole-blood samples (n = 6) were perfused over a microchip coated with collagen and tissue thromboplastin at different shear rates, and thrombus formation was quantified by measuring flow pressure changes. For comparison, rotational thromboelastometry (ROTEM) was used to evaluate fibrinolytic activity under static conditions.
Results: At a shear rate of 240 s^{-1}, t-PA (200–800 IU/ml) concentration-dependently delayed capillary occlusion, whereas at 600 s^{-1}, capillary occlusion was significantly faster and t-PA had limited effects, even at a supra-pharmacological concentration (800 IU/ml). In contrast, 200 IU/ml t-PA efficiently prevented clot formation in the ROTEM assay. The combined treatment of blood with a specific PAI-1 inhibitor (PAI-039) moderately enhanced the efficacy of t-PA, but only under flow conditions. In addition, 1:1-diluted blood samples of PAI-1-deficient (−/−) mice showed a significant delay of capillary occlusion at 240 s^{-1}, compared with those from wild-type mice (1.55 fold; $P < 0.001$). This delayed occlusion was reproduced in samples containing platelets from PAI-1 −/− and plasma from wild type, but was not observed by the opposite combination of blood components.
Conclusions: The present results suggest that the anti-thrombotic efficacy of t-PA is sensitive to arterial shear flow, and that PAI-1 secreted from activated platelets plays an essential role in thrombolytic resistance.

1. Introduction

Thrombolytic therapy with tissue plasminogen activator (t-PA) is frequently used for the treatment of patients with acute ischemic stroke. However, despite the proven benefits for reducing mortality and disability after ischemic stroke [1–3], therapeutic failure still occurs at a high rate, due to thrombolysis resistance, particularly in arterial circulation.

Under physiological conditions, resistance to thrombolysis and the risk of thrombosis are increased when plasma levels of plasminogen activator inhibitor type 1 (PAI-1), which is the primary inhibitor of both t-PA and urokinase type PA (u-PA), are elevated. Plasma levels of PAI-1 can be increased by both hereditary factors, particularly gene polymorphism [4–6], and by acquired reasons, such as metabolic syndrome [4–6]. PAI-1 is also present in the α-granules of platelets and is released in response to platelet-activating stimuli [7,8]. Previous studies using animal thrombosis models have shown that platelet-rich arterial thrombi contain high levels of PAI-1 [9] and are more resistant to lysis by t-PA [10]. An in-vitro experiment using chandler loop also proved that the head of the thrombi contained both higher platelets count and higher PAI-1 concentration than those in the tail of the thrombi formed under arterial condition. In addition, recanalization of arterial thrombi in PAI-1-deficient (−/−) mice is increased by t-PA infusion at

Abbreviations: AUC_{30}, area under the flow pressure curve; CFT, clot formation time; CT, clotting time; CTI, corn trypsin inhibitor; MCF, maximum clot firmness; ML, maximum lysis; OT, occlusion time; PAI-1, plasminogen activator inhibitor type 1; ROTEM, rotational thromboelastometry; T_{10}, time to 10 kPa; TEG, thromboelastography; TF, tissue factor; t-PA, tissue plasminogen activator; u-PA, urokinase type PA; WT, wild-type.

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pharmacological concentrations compared to wild-type (WT) mice [11]. Together, these findings suggest that PAI-1 present in plasma and/or platelets likely contributes to arterial thrombolysis resistance and thrombogenesis, although the underlying mechanisms remain unclear.

In humans, PAI-1 deficiency is reported to cause life-threatening hemorrhage, suggesting that PAI-1 is also a key modulator of human hemostatic processes, although PAI-1 (−/−) mice do not display an increased propensity for bleeding [12,13]. These phenotypic differences associated with PAI-1 deficiency between species limits the direct application of animal models for analyzing the mechanisms underlying the regulation of t-PA activity by plasma and platelet PAI-1 in humans. In addition, current in-vitro assays to analyze fibrinolytic reactions, such as clot-lysis tests [14], thromboelastography (TEG) and rotational thromboelastometry (ROTEM) [15], are generally performed in the absence of blood flow, which limits their relevance for pathologic arterial thrombosis and physiological hemostasis.

To overcome these limitations, we employed a recently developed automated microchip flow chamber system to mimic arterial circulation [16] and evaluated the influence of shear stress on both platelet-rich thrombus formation and lysis. Thrombolytic efficacies of t-PA were measured in the presence and absence of PAI-039, a specific PAI-1 inhibitor, at shear rates of 240 and 600 s^{-1}, which simulate shear flows in large and small/medium sized arteries, respectively. ROTEM measurements were also performed to comparatively analyze the thrombolytic effects of t-PA under static conditions. The obtained results suggested that resistance to t-PA-evoked thrombolysis was sensitive to arterial shear flow and that PAI-1 secreted from activated platelets plays an essential role in thrombolysis resistance and thrombogenesis.

2. Materials and methods

2.1. Materials

The microchips used in the flow-chamber system experiments were manufactured by Richell Corp. (Toyama, Japan) (Supplemental Fig. 1A). Porcine type I collagen was purchased from Nitta Gelatin, Inc. (Osaka, Japan). Tissue thromboplastin was purchased from Sysmex (Hyogo, Japan). Corn trypsin inhibitor (CTI) was prepared as reported previously [17].

t-PA (alteplase) and urinary plasminogen activator (u-PA; uronase) were purchased from Tanabe Mitsubishi Pharma (Tokyo, Japan) and Mochida Pharma (Tokyo, Japan), respectively. PAI-039, a specific PAI-1 inhibitor, was purchased from Axon Medchem BV (Groningen, The Netherlands). Recombinant tissue factor (r-TF) was purchased from Mitsubishi Chemical Medience (Tokyo, Japan). Fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CD41 immunoglobulin G (IgG) and FITC-conjugated mouse IgG were purchased from Beckman Coulter (Miami, FL, USA). Rabbit anti-human fibrinogen IgG was purchased from Dako (Tokyo, Japan). Normal rabbit IgG was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Alexa594 was obtained from Invitrogen (Carlsbad, CA, USA). All other reagents were obtained from Wako Pure Chemicals.

2.2. Blood samples

Blood samples from 6 healthy, fasting volunteers (4 males, 2 females; mean age 33.83 ± 4.92 years) were collected in plastic tubes containing 3.2% sodium citrate (Terumo, Tokyo, Japan). The subjects had not taken any medication that might affect platelet function or coagulation in the two weeks preceding the blood collection. The study protocol was approved by the local ethics committee of Kinki University (Osaka, Japan), and informed written consent was obtained from all individuals prior to their participation.

2.3. Microchip-based flow chamber measurements of thrombus formation

To analyze thrombus formation under flow conditions, we utilized a microchip-based flow chamber system (Total Thrombus-formation Analysis System [T-TAS]; Fujimori Kogyo Co., Ltd., Tokyo, Japan). The T-TAS system is equipped with a pneumatic pump, flow pressure sensor, and videomicroscope, and is capable of measuring thrombus formation under adjustable shear rates, as described previously (Supplemental Fig. 1B) [16]. For the analysis, citrated whole blood (480 μl) containing various concentrations of t-PA, u-PA, PAI-039, or their combinations was mixed with 20 μl a 300 mM $CaCl_2$ solution containing 1.25 mg/ml CTI (final concentrations: 12 mM $CaCl_2$ and 50 μg/ml CTI). After mixing, each blood sample was immediately perfused over a microchip coated with collagen and tissue thromboplastin (Supplemental Fig. 1A) at flow rates of 4 and 10 μl/min, which create initial wall shear rates of normal small veins (240 s^{-1}) and of medium-sized arteries (600 s^{-1}), respectively, as estimated by the FLUENT program (Ansys Co., Ltd., Tokyo, Japan) [18]. Flow pressure changes were monitored by the pressure transducer located upstream of the microcapillaries during the perfusion experiments. Thrombus formation and breakdown within the microcapillaries cause flow disturbances that result in pressure increases and decreases, respectively.

The obtained flow pressure pattern for each sample was used to analyze thrombus formation based on the following estimated parameters: time to 10 kPa (T_{10}; min), which is the time required to reach 10 kPa from the baseline pressure and reflects the onset of thrombi formation; occlusion time (OT; min), which is the time required to reach 80 kPa from the baseline pressure and reflects nearly complete capillary occlusion; and area under the flow pressure curve for 30 min (under 80 kPa) after the start of the assay (AUC_{30}), which reflects total thrombogenicity.

2.4. Thromboelastometry measurements

The ROTEM system (TEM International, Munich, Germany) was used to analyze clot formation in recalcified whole blood under static conditions. Briefly, citrated whole blood (300 μl) containing an appropriate concentration of t-PA, u-PA, PAI-039, or their combinations was mixed with 20 μl Star-TEM ($CaCl_2$; final concentration, 12 mM) in the analyzer cup, which was heated at 37 °C.

The following ROTEM measurements were performed over a 60-min period: clotting time (CT; sec), which corresponds to the lag time before clotting; clot formation time (CFT; sec), which reflects the initial rate of clot formation; maximum clot firmness (MCF; mm), which is a measure of the maximal tensile strength of the clot; and maximum lysis (ML; %), which is the ratio of clot firmness lost during the measurement (Supplemental Fig. 2).

2.5. Confocal laser scanning microscope analysis of thrombi

For the analysis of thrombi by a confocal laser scanning microscopy, thrombi formed on the coated microchip surface were immediately washed three times with phosphate-buffered saline (PBS) and then incubated with FITC-conjugated mouse anti-human CD41 (platelet GPIIb) IgG (1:5 dilution) for 15 min in the dark. After three washes with Tris-

Table 1
Characteristics of blood samples from healthy volunteers.

	Mean ± SE	Max	Min
PT (sec)	11.2 ± 0.2	11.7	10.3
APTT (sec)	28.8 ± 0.7	31.4	26.5
Hematocrit (%)	43.3 ± 2.1	50.2	37.7
Platelet (10^4/μl)	25.2 ± 1.1	28.6	22.2
Total-PAI-1 (ng/ml)	17.3 ± 3.4	30	10
Fibrinogen (mg/dl)	225.5 ± 16.2	289	169
Plasminogen (%)	96.8 ± 5.0	111	75