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Direct microscopic monitoring of initial and dynamic clot lysis using plasmin or rt-PA in an *in vitro* flow system

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ABSTRACT

Introduction: Plasmin is a direct-acting thrombolytic agent with a favorable safety profile upon intra-arterial delivery in pre-clinical and phase I studies. However, the thrombolytic efficacy of plasmin, relative to that of rt-PA, remains to be established. We have compared the dynamics of clot lysis with plasmin or rt-PA in an *in vitro* perfusion system, in which thrombolytic agent is administered locally, allowed to induce lysis for short intervals, then washed with plasma in a re-circulation circuit.
Materials and Methods: Whole blood human clots were prepared in observation chambers, exposed to plasmin or rt-PA at equimolar concentrations (1.2/1.0, 1.8/1.5 and 2.4/2.0 mg/ml) for measured intervals of time, followed by perfusion with human plasma. Clot size was monitored by digital analysis of sequential photographs obtained through an optical microscope.
Results: Plasma perfusion after incubation with thrombolytic agent rapidly removed superficial clot fragments. This initial decrease in clot size was greater with plasmin than with rt-PA when tested at the highest concentrations of agent (0.63 ± 0.11 vs. 0.30 ± 0.11, $p = 0.001$ for clots with non-cross-linked fibrin and 0.53 ± 0.15 vs. 0.14 ± 0.15, $p = 0.02$, for clots with cross-linked-fibrin). Subsequent clot lysis during plasma flow was greater after prior incubation with rt-PA. Longer incubation times of plasmin resulted in larger portions of the clot being washed free. Repeated plasmin incubations and plasma perfusions of a clot successfully induced stepwise reductions in clot size.
Conclusions: Initial clot lysis is greater with direct exposure using plasmin than rt-PA. During washout and circulation with plasma, rt-PA induced continued clot lysis, while plasmin lysis was curtailed, presumably because of plasmin inhibition.

Introduction

Locally administered plasminogen activator is an important aspect of catheter-directed recanalization therapy for peripheral arterial and graft occlusion and for deep vein thrombosis. Recombinant tissue plasminogen activator (rt-PA) has been the thrombolytic agent of choice for decades, achieving good therapeutic efficacy, but with a risk of bleeding complication, most severely expressed as symptomatic intracranial hemorrhage [1]. The concept of locally-administered thrombolysis with the direct thrombolytic enzyme, plasmin, was introduced in an animal model in 2001 [2], and within a decade successfully progressed through two phase I, dose-escalation safety clinical trials [3,4]. The potential clinical advantage of plasmin over rt-PA is its hemostatic safety, which stems from the presence of plasmin inhibitor, α_2-antiplasmin in human plasma at about 1 mmol/l (70 mg/l) [5], which inactivates circulating plasmin after local administration. There are several rt-PA inhibitors present in human plasma, PAI-1, PAI-2, protease nexin and PAI-3, of which the major inhibitor is plasminogen activator inhibitor-1 (PAI-1) [6]. PAI-1 is present in human plasma in nanomolar concentrations (about 10–20 mg/l), which are insufficient to neutralize administered rt-PA [7]. Thus, rt-PA remains active to continue its thrombolytic effect, but may lyse hemostatic plugs at sites of vascular injury [1].

Abbreviations: FOV, filed of view; PAI-1, plasminogen activator inhibitor-1; RMS, root mean square; rt-PA, recombinant tissue plasminogen activator.

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In contrast to its well-defined safety profile, plasmin has not been fully characterized for its the therapeutic efficacy. Plasmin has shown good efficacy in animal models of thrombosis [8,9] and was equivalent to rt-PA in lysis of ex-vivo cerebral thromboemboli [2], but a comparison with rt-PA has not heretofore been studied in an in vitro flow system of clot lysis. It is possible that plasmin has better thrombolytic effect than rt-PA in a static situation, but a decreased effect in a model with plasma flow, due to termination of its action after contact with antiplasmins (α_2-antiplasmin and α_2-macroglobulin).

The aim of this study is to compare the dynamics of clot lysis with plasmin and rt-PA in a novel *in vitro* perfusion system, where incubation of agent with clot in a perfusion chamber mimics local administration, and a follow-up period of tangential plasma flow along the clot mimics partial vessel recanalization. Our test system allows continuous monitoring of clot lysis by microscopic imaging and repeat observations of local and circulating phases of clot lysis.

Materials and Methods

Clot Preparation and Flow Apparatus for Clot Lysis

After informed consent, blood was collected before each set of experiments from the cubital vein of a single healthy volunteer into a 4.5 ml vial (Vacuntainer, Becton-Dickinson, Germany) containing 0.45 ml of 0.129 mol/l Na-citrate. Clots were prepared from 100 µl of blood mixed with 10 µl of thrombin (Thrombin, Sigma, Germany, final concentration 1.37 IU/ml) with or without adding 10 µl of 2 M $CaCl_2$. The mixture was injected into an observation chamber made of optical microscopy slides (Fig. 1a), length 25 mm along the direction of flow, width 5 mm and height 1 mm [10]. During clot formation which proceeded from liquid blood to a gel-like state in about 1 min, and during the subsequent 90 – 120 min of final clot formation at room temperature (24 °C), the chambers were tilted by 30° so that wedge-shaped clots were obtained, occupying 10% of the chamber volume (Fig. 1b). During clot dissolution experiments, the observation chamber was connected to a plasma reservoir containing 30 ml of human plasma, circulated at 37 °C by peristaltic pump (LKB Bromma HPLC 2249,Uppsala, Sweden) (Fig. 1c).

Cross linking of fibrin in non-recalcified and recalcified blood clots was checked by measuring D-dimer by the Biopool Auto-Dimer® turbidimetric latex agglutination method (TrinityBiotech, Lemco, Germany) using the BCT analyzer (Dade Behring, Siemens AG, Munich, Germany) in the supernatant of the lysate of both types of clots exposed to rt-PA (Actilyse, BoehringerIngelheim GmbH, Ingelheim am Rhein, Germany) at 2.0 mg/ml for 10 min. The experiment was performed in triplicate.

Clot Lysis

We exposed clots from non-recalcified blood to plasmin (80 kDa) at 1.2, 1.8 and 2.4 mg/ml or to rt-PA (67 kDa) at equimolar concentrations of 1.0, 1.5 and 2.0 mg/ml, respectively. Clots from recalcified

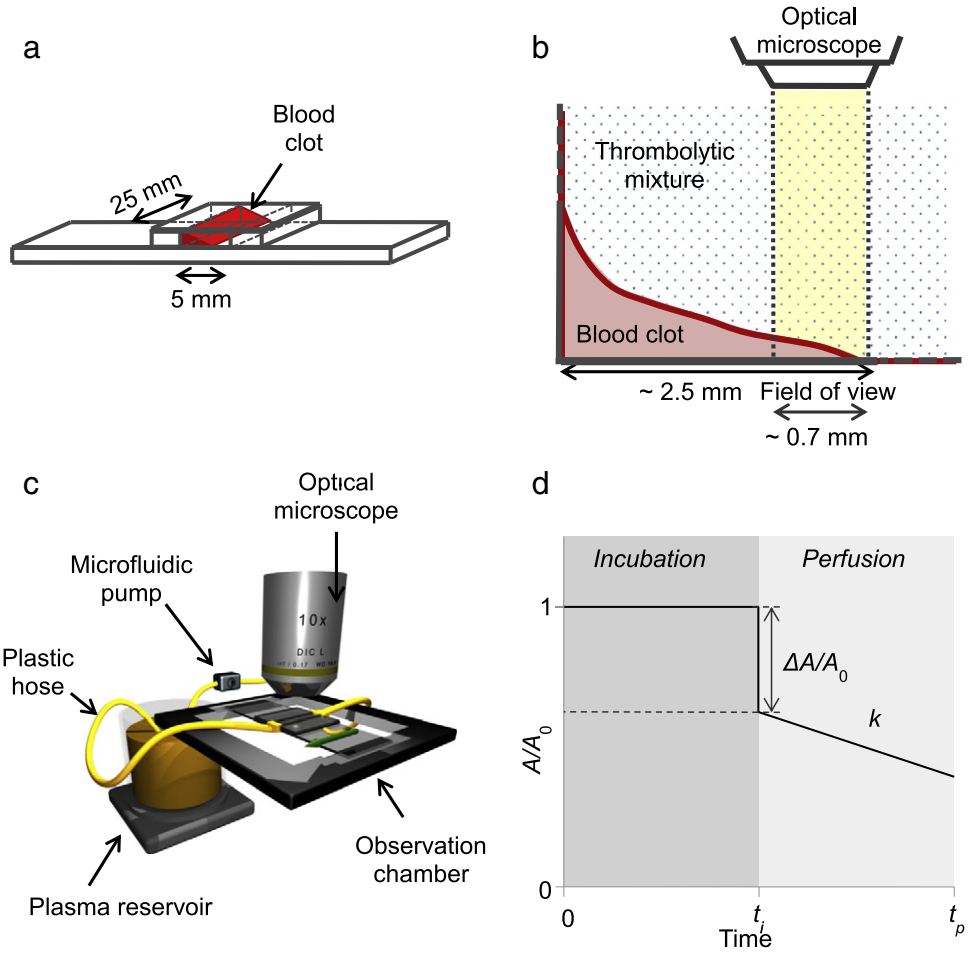

Fig. 1. Observation chamber containing a model blood clot (a), schematic presentation of the wedge-shaped blood clot (b), experimental setup (c) and schematic presentation of the dissolution process as a function of time (Eq. (1)) (d). The observation chamber was connected to a plasma reservoir and microfluidic pump using plastic hoses. Dissolution dynamics of blood clots was monitored by an optical microscope. Schematic presentation of the dissolution process suggests that the initial relative clot area is equal to unity until the end of incubation (time t_i). This is followed by an abrupt decrease of relative clot area $\Delta A/A_0$ at the beginning of perfusion phase. Subsequently, the dissolution curve reverts into a more gradual clot area decrease with a slope k until the end of perfusion (time t_p).