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# Microscopic clot fragment evidence of biochemo-mechanical degradation effects in thrombolysis

Franci Bajd [a], Jernej Vidmar [b], Aleš Blinc [c], Igor Serša [a,*]

[a] Jožef Stefan Institute, Jamova 39, Ljubljana 1000, Slovenia
[b] Institute of Physiology, Medical Faculty, University of Ljubljana, Zaloška 4, Ljubljana 1000, Slovenia
[c] Department of Vascular Diseases, University of Ljubljana Medical Centre, Zaloška 7, Ljubljana 1525, Slovenia

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ABSTRACT

*Introduction:* Although fibrinolytic treatment has been used for decades, the interactions between the biochemical mechanisms and the mechanical forces of the streaming blood remain incompletely understood. Analysis of the blood clot surface *in vitro* was employed to study the concomitant effect of blood plasma flow and recombinant tissue plasminogen activator (rt-PA) on the degradation of retracted, non-occlusive blood clots. Our hypothesis was that a faster tangential plasma flow removed larger fragments and resulted in faster overall thrombolysis.

*Materials and Methods:* Retracted model blood clots were prepared in an optical microscopy chamber and connected to an artificial perfusion system with either no-flow, or plasma flow with a velocity of 3 cm/s or 30 cm/s with or without added rt-PA at 2 μg/ml. The clot surface was dynamically imaged by an optical microscope for 30 min with 15 s intervals.

*Results:* The clot fragments removed during rt-PA mediated thrombolysis ranged in size from that of a single red blood cell to large agglomerates composed of more than a thousand red blood cells bound together by partly degraded fibrin. The average and the largest discrete clot area change between images in adjacent time frames were significantly higher with the faster flow than with the slow flow (14,000 $\mu m^2$ and 160,000 $\mu m^2$ vs. 2200 $\mu m^2$ and 10,600 $\mu m^2$).

*Conclusions:* On the micrometer scale, thrombolysis consists of sequential removal of clot fragments from the clot surface. With increasing tangential plasma flow velocity, the size of the clot fragments and the overall rate of thrombolysis increases.



## Introduction

Formation of intravascular blood clots is an important component of venous thromboembolism [1] and of the final stages of atherothrombosis that may, depending on the affected vascular bed, cause acute coronary syndrome, ischemic cerebrovascular stroke [2,3], or critical limb ischemia [4]. Thrombotic disorders may be treated by thrombolytic therapy with plasminogen activators that convert the inactive proenzyme plasminogen into the active serine protease plasmin which in turn degrades the fibrin meshwork [5]. Although thrombolytic therapy has been used for decades, the interactions of its biochemical mechanisms with the mechanical forces of streaming blood remain incompletely understood.

Experimental studies and mathematical models have been published on dissolution of non-occlusive blood clots with emphasis on the dynamics of the thrombolytic biochemical reactions [6–10], or on the mechanical effects of the streaming blood on clot degradation [6,8,11]. A common finding of these studies is that fast tangentially directed blood flow along a non-occlusive blood clot accelerates thrombolysis. The proposed mechanisms of tangential blood flow are to increase the delivery of the thrombolytic agent into the blood clot and to exert large mechanical forces on the clot surface due to blood viscosity or turbulence [11–13]. Additionally, it was reported that, in comparison to non-retracted blood clots, retracted ones are less susceptible to thrombolysis due to syneresed serum from their porous interior [13–15]. All of the above mentioned experimental studies employed either an artificial perfusion system combined with a macroscopic imaging technique (MRI, conventional photography …) or were done in the absence of flow (static conditions) and employed microscopic imaging (conventional optical and confocal microscopy). Few studies combined perfusion experiments with microscopic imaging [7]. However, none of the studies was focused on the analysis of clot degradation products in relation to the velocity of tangential blood plasma flow.

Our previous studies have shown that flow enhances thrombolysis to a degree that cannot be ascribed only to promotion of biochemical clot degradation [6,15]. We hypothesized that with faster tangential flow, resulting in stronger shear forces of the streaming plasma, the fragments removed from the clot surface would be larger, albeit

*Abbreviations:* RBC, red blood cell; rt-PA, recombinant tissue plasminogen activator.

* Corresponding author. Fax: +386 1 477 3696.
*E-mail address:* igor.sersa@ijs.si (I. Serša).

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incompletely biochemically degraded. The aim of this study was to test this hypothesis by optical microscopy of the surface of retracted model blood clots in an artificial perfusion system.

## Materials and methods

### *Model blood clots*

Whole-blood was collected from the cubital veins of healthy volunteers, who showed no evidence of coagulation disorders or acute illness. Blood was drawn under standardized conditions into 4.5 ml-vials (Vacuntainer, Becton-Dickinson, Germany) containing 0.45 ml of 0.129 mol/l Na-citrate. To neutralize the anticoagulation effect of Na-citrate, 50 µl of $CaCl_2$ at a concentration of 2 mol/l, was added to the collected blood samples. Clotting was initiated by adding 100 µl/ml of thrombin (Thrombin, Sigma, Germany) to a final concentration of 1.37 NIH unit/ml. This was followed by the injection of 25 µl of the mixture into a chamber, designed for observation by optical microscopy (Fig. 1A). The observation chamber, which was made of microscopy slides, was 25 mm long (dimension along the flow direction), 5 mm wide and 1 mm high. To obtain clots with a gradually decreasing thickness, i.e., a triangular profile, clotting was performed in an observation chamber tilted 30° along the axis parallel to the longest side of the chamber. The adherence of the clot to the chamber side wall was assured by coating the wall with an epoxy two-component glue (Uhu, Germany) (Fig. 1B). The glue did not interfere with clotting or thrombolysis. A similar type of glue was used for attaching clots to the perfusion system in our previous work without marked interference with thrombolysis [15]. The blood mixture in the chamber was incubated for two hours at room temperature (24 °C) to allow for complete formation of the fibrin meshwork and for spontaneous clot retraction. After the incubation, the initial wedge-shape of blood clot was slightly deformed due to surface tension and clot retraction. Each clot in the study was prepared in a new, identically designed observation chamber.

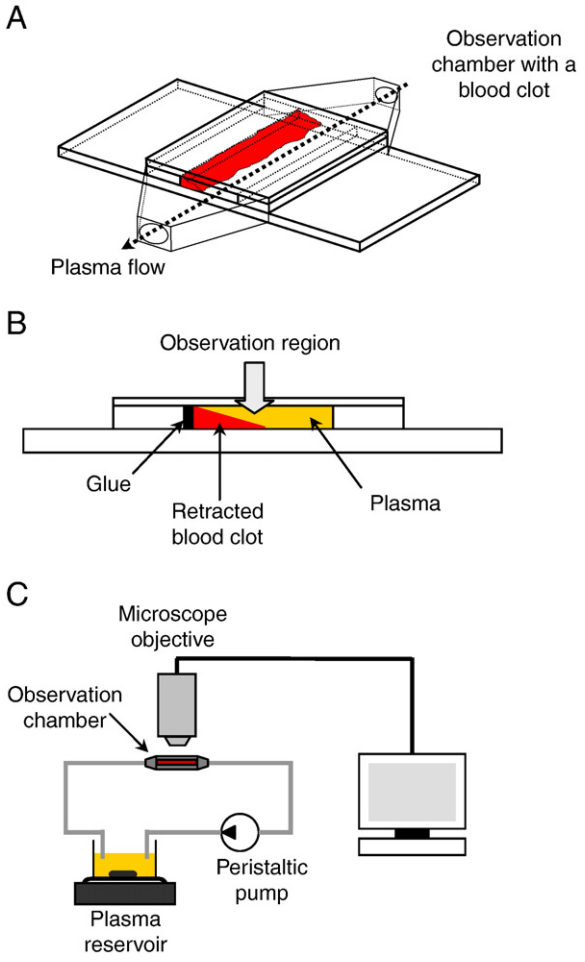


**Fig. 1.** Optical microscopy of thrombolysis was performed with a model retracted whole-blood clot tightly sealed in the observation chamber (A). A cross-section through the chamber (B) shows the chamber composition; a thin layer of the wedge-shaped blood clot and flowing plasma above it. The chamber was connected to the perfusion system and placed under the optical microscope equipped with a digital camera (C).

### *Conditions of thrombolysis*

The clot filled 20% of the chamber volume while 80% of the volume was available for plasma flow. Cone shaped, 5 mm long plastic inserts were glued by an epoxy two-component glue to the proximal and distal sides of the observation chamber and connected at both ends to a flexible plastic hose of 3 mm inner diameter. The inserts were needed to transform the flow profile from the cylindrical geometry in the hose to the planar geometry in the chamber. The 1 m long hose connected the observation chamber to a peristaltic pump (913 Mityflex, Germany) in a thermoregulated (36 °C) reservoir containing 40 ml of human plasma of a matching AB0 blood group (Fig. 1C). Fresh frozen plasma was obtained from the Blood Transfusion Centre of the Republic of Slovenia that processes exclusively blood donated by volunteers who pass the clinical and laboratory health standards and do not take medication. Plasma from single donors was packed in bags of approximately 250 ml. Prior to dissolution experiments the frozen plasma contained in a single bag was thawed and used in six individual experiments. In total plasma of five different donors was distributed between 30 experiments. Each experiment started with an initial perfusion of the clot, prior to thrombolysis, for 15 min at a flow rate of 0.15 ml/s in order to remove any possible debris weakly attached to the clot surface. Perfusion was continued for an additional 30 min in one of the six different experimental regimes: non-perfusion, slow perfusion (volume flow of 0.15 ml/s with a velocity of 3 cm/s) and faster perfusion (volume flow of 1.5 ml/s with a velocity of 30 cm/s) all with or without the thrombolytic agent. Recombinant tissue plasminogen activator - rt-PA (Actilyse, Boehringer, Germany) was used at a concentration of 2 µg/ml, which corresponds to the expected plasma concentration in an average-sized person after injection of a 10 mg rt-PA bolus, i.e., during treatment of acute myocardial infarction [16]. At least five experiments were performed for each of the six experimental regimes.

### *Optical microscopy*

Clots were imaged by a Nikon 80i Eclipse optical microscope equipped with a Nikon 10x Plan Fluor objective (numerical aperture 0.3, working distance 16 mm) and a high-resolution CCD camera (Nikon DS-Fi1). The optical system was controlled by the Nikon NIS Elements software package. Digital images were recorded dynamically at 15 s intervals (0.07 fps) with a 20 ms exposure time during continuous flow of plasma through the chamber. The imaging matrix was $640 \times 480$ with an in-plane resolution of 1.3 µm per pixel that yielded a field of view (FOV) of $832 \times 624$ µm. The optical microscopy settings represented the best compromise between the relatively large FOV and the resolution needed for detection of single whole-blood cells and larger clot degradation products. The FOV was positioned above the border between the clot and the glass. Initially, approximately 90% of the FOV was covered by the thin clot region with flowing plasma above.

### *Image analysis*

To obtain clot dissolution curves (normalized non-lysed blood clot area as a function of time) and to obtain the size distribution of