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Clot penetration and retention by plasminogen activators promote fibrinolysis

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ABSTRACT

Tissue-type plasminogen activator (tPA) remains the sole thrombolytic approved by the FDA for the treatment of pulmonary embolism (PE). tPA has not been replaced by third generation plasminogen activators, *e.g.* Reteplase (Ret) and Tenecteplase (TNK) that circulate with longer life-spans and in theory should have more extended potency *in vivo*. One reason for this paradox is the inability to assign units of activity to plasminogen activators based on specific biologically relevant standards, which impairs objective comparison. Here, we compare clot permeation, retention and fibrinolytic activities of tPA, TNK and Ret *in vitro* and clot composition over time with outcome in a mouse model of disseminated pulmonary microembolism (ME). When clots were incubated in the continuous presence of drug, tPA, TNK and Ret lysed fibrin clots identically in the absence of PA inhibitor-1 (*e.g.* PAI-1). Ret, which has lower fibrin affinity and greater susceptibility to inhibition by PAI-1 than tPA, was less effective in lysing plasma clots, while TNK was less effective when the fibrin content of the clots was enhanced. However, when clots were afforded only brief exposure to drug, as occurs *in vivo*, Ret showed more extensive clot permeation, greater retention and lysis than tPA or TNK. These results were reproduced *in vivo* in a mouse model of ME. These studies indicate the need for more relevant tests of plasminogen activator activity *in vitro* and *in vivo* and they show that clot permeation and retention are important potential predictors of clinical utility.

1. Introduction

Pulmonary embolism (PE) is associated with a mortality estimated to range from 1 to 15% in patients deemed to be at low or intermediate risk, and the incidence doubles in haemodynamically unstable patients [1,2]. Treatment of PE is challenging, in part due to difficulties in diagnosis [3], which may delay drug administration until clots have undergone considerable extension into more distal pulmonary vasculature. Fibrinolytic agents, such as recombinant tissue-type plasminogen activator (tPA), urokinase and streptokinase are used for thrombolysis in the setting of massive PE, whereas in less severe cases, heparin alone is generally chosen, less for superior efficacy than to avoid the risk of hemorrhage [4].

Third generation plasminogen activators, Reteplase (Ret) and Tenecteplase (TNK), were developed to overcome the disadvantages of tPA (*e.g.*, short circulation time and rapid inactivation by plasma inhibitors, including PAI-1, which dictates the need for using high doses). The circulation times of Ret and TNK are an order of magnitude longer than tPA. However, Ret, lacking the finger, EGF-like and kringle-1 domains, has lower fibrin (Fn) affinity and less resistance to PAI's [5,6]. In contrast, TNK was created by introducing point mutations into three regions of tPA that prolongs its circulation time without impairing fibrin affinity and increases its resistance to PAI's [7,8]. Notwithstanding their longer lifespans and their different susceptibilities to inhibition, there have been few clinical studies comparing their efficacy with tPA [4,6,9]. Such comparisons are complicated by a lack of a uniform and clinically relevant definition of units of activity [10]. Moreover, these newer plasminogen activators have not displaced tPA in the treatment of PE and other thromboembolic diseases [11–15].

Nevertheless, there is some evidence to support the advantage of Ret in the treatment of PE [6], notwithstanding its apparent inferior plasminogen activator potency compared with tPA [5].

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Here we sought to identify the *in vitro* characteristics of plasminogen activators that correlate most closely with *in vivo* activity. In order to rigorously compare PA, we used equimolar drug concentrations, which enabled us to harmonize dosing. Our *in vitro* results demonstrate that tPA, TNK and Ret share equal plasminogen activator potency in the absence of plasma and with prolonged clot exposure, but diverge in their capacity to lyse plasma clots due to differences in clot permeation, retention and susceptibility to plasma inhibitors. Indeed, Ret demonstrates fibrinolytic supremacy *in vitro* and *in vivo* in settings where PA-clot interactions are restricted by rapid diffusion and therefore rely upon clot retention. These features lead to more extensive lysis of thrombi emanating from both the clot surface and from within the clot structure itself.

2. Methods

2.1. Reagents, samples and animals

Thrombin, bovine fibrinogen (Fg) and trichloroacetic acid were from Sigma–Aldrich, Madrid, Spain. Potato carboxypeptidase inhibitor (PCI) was from Calbiochem, Madrid, Spain. Plasminogen activator inhibitor-1 (PAI-1) was from American Diagnostica, Pfungstadt, Germany. Alteplase, tPA (Activase®) and Tenecteplase, TNK (TNKase®) were from Genentech, San Francisco, CA, USA. Reteplase, Ret (Retavase®) was from Centocor, Philadelphia, PA, USA. Dy-light 633-NHS, Fluorescein-NHS and Iodogen® were from Pierce Biotechnology Inc., Rockford, IL, USA. [^{125}I]Na was from Perkin-Elmer, Madrid, Spain. Ketamine (Imalgene®) was from Merial Laboratories, Tarragona, Spain; and Xilacine (Rompun®) from Bayer España, Barcelona, Spain. Mice (C57Bl6/J) were from Charles River Laboratories, Barcelona, Spain. Human platelet poor plasma (PPP) was isolated from whole blood donated by healthy volunteers at the "Hospital Universitario Príncipe de Asturias" (Alcalá de Henares, Madrid, Spain) and the Regional Center for Blood Transfusion (Comunidad de Madrid, Madrid, Spain) with informed consent following an approved protocol.

2.2. Protein modification

Proteins were radiolabeled with [^{125}I]Na using Iodogen® (Pierce) according to the manufacturer's instructions or fluorescently labeled with Dy-light 633-NHS (in the case of PA) or Fluorescein-NHS (in the case of Fg) through their free amino groups following incubation with 20× molar or 10× molar dye, respectively. The residues of Dy-light 633 incorporated per molecule of PA were comparable for all PAs (0.5–0.7 residues of fluorophore per molecule), with no alteration in their fibrinolytic capacity (data not shown). In the case of Fg, trace amounts of Fluorescein-Fg were added to the final solution before addition of $CaCl_2$ and thrombin with no affect on clotting compared to unlabeled Fg.

2.3. In vitro *studies*

Fibrin clots (PFC) were prepared by mixing a solution containing bovine Fg in Krebs Ringer Buffer (KRB) with $CaCl_2$ and thrombin (20 mM and 0.2 U/ml final concentrations, respectively). Two concentrations of Fg were employed: (a) 3 mg/ml (physiological) for assays involving continuous exposure to PAs and (b) 10 mg/ml (Fg enriched clots) for shorter exposures after which clots were washed to remove unbound PAs. Trace amounts of [^{125}I]Fg were added in some experiments to quantify lysis as previously described[16] using clots that had been allowed to polymerize and mature for 20 min at room temperature. Plasma clots (PPP) were formed by adding $CaCl_2$ and thrombin to human plasma and were then allowed to polymerize and mature as above. To examine the role of TAFI and in experiments involving confocal microscopy, plasma clots were enriched with 3 mg/ml bovine Fg to generate a more stable clot that would withstand washing and simulates the higher fibrin content associated with several pathophysiological conditions [17,18].

2.4. Constant PA exposure

[^{125}I]PFCs were overlaid with 0.5–2 nM PA in KRB. These PA concentrations are sufficient to be clinically relevant, but low enough to avoid the plasminogen "steal" effect [19]. To study the inhibitory effect of PAI-1, each PA was preincubated at specified molar ratios of reactants for 15 min at 4 °C. To initiate fibrinolysis, clots were incubated at 37 °C and the radioactivity released into the supernatants was measured at the indicated times. To eliminate the inhibitory effect of TAFI in plasma, a 70× molar excess of PCI (30 μg/ml) was added to a 200 μl volume of [^{125}I]PPP enriched in Fg prior to adding $CaCl_2$ and thrombin. After the clot was allowed to mature, specified concentrations of each PA were added in the presence of 70× molar excess PCI, and fibrinolysis was measured as described above.

2.5. Transient PA exposure

2.5.1. PA fibrinolysis

PFC were enriched in Fg (10 mg/ml) and overlaid with equimolar concentrations of each PA in KRB for 5 min at 4 °C. The clots were then washed with ice-cold buffer, transferred to a new tube and overlaid with fresh KRB. Fibrinolysis proceeded at 37 °C.

2.5.2. PA retention

Unlabeled PFC were incubated with [^{125}I]PA for 5 min at 4 °C, washed 3–5 times in 3 ml ice-cold KRB and the residual clot-associated radioactivity was measured.

2.5.3. PA penetration

Clots were formed in a hand-made chamber composed of a microscope glass and its cover-slide, separated by thin plastic spacers, leaving one side open for addition of Fg, plasma or PA. The chambers were mounted using cyanoacrylate glue, providing a final inner volume of 1.5 mm × 2 cm × 2 cm. The chamber was then filled with a mixture of 3 mg/ml Fluorescein-Fg, either in KRB (PFC) or in human plasma (PPP), $CaCl_2$ and thrombin before polymerization. The clots were allowed to polymerize and mature for 20 min at room temperature and were subsequently kept at 4 °C throughout the experiment. Clots were then overlaid with equimolar concentrations of Dy-light 633-PAs for 5 min, washed 3–5 times with cold KRB, and drug retention and penetration was analyzed by spectral confocal microscopy with a TC SP5 Leica Microsystem using a 40x immersion objective (HCX PL 1.25 oil). Penetration distances were quantified using the LAS-AF® Leica application suite software.

2.6. Pulmonary embolism model

To assess biologic activity *in vivo*, we used a previously developed murine model of PE mediated by human plasma microemboli (ME). Briefly, a 200 μl suspension of 1.5–5 μm [^{125}I]ME was injected into the jugular vein. ME distribute homogenously throughout the pulmonary circulation within 5–10 min, occluding small capillaries, and then enlarge *in situ* to occlude mid-size lung arteries[20,21]. Various concentrations of PAs were injected into the contralateral jugular vein 10 min post [^{125}I]ME. Animals were sacrificed 1 hr after the initiation of the experiment. Lungs were extracted, rinsed in saline, and residual