Comparison of Plasmin with rt-PA in Lysis of Cerebral Thromboemboli Retrieved from Patients with Acute Ischemic Stroke

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Abstract

Background and purpose—Plasmin is a direct-acting thrombolytic with a better safety profile than recombinant tissue-type plasminogen activator (rt-PA) in animal models. With the application of retrieval devices for managing acute ischemic stroke, extracted thromboemboli are available for 

ex vivo

examination. We ask whether such thrombi are amenable to plasmin thrombolysis and whether such activity is different with rt-PA.

Methods—Thromboembolic fragments (total 29) were retrieved from the intracranial carotid artery system of 15 patients with acute ischemic stroke and randomly assigned to 

ex vivo

thrombolysis with plasmin or rt-PA. After an initial 2-hour exposure, residual material was exposed to the other agent for an additional 2 hours. Thrombolysis was quantified by change in thrombus area and released D-dimer.

Results—Plasmin induced significant 

ex vivo

thrombolysis of cerebral arterial thromboemboli, decreasing area by 45.9 +/− 29.4% and 69.2 +/− 52.5% and inducing median D-dimer release of 108,180 μg/L (range 16,780 - 668,050 μg/L) and 16,905 μg/L (range 240 - 403,085 μg/L) during the first and second 2-hour incubation periods, respectively. These changes were not different from those obtained with rt-PA, which decreased area by 34.7 +/− 57.8% (P=0.63) and by 68.4 +/−...
26.9% (P=0.97), and induced median D-dimer release of 151,990 µg/L (9,870 - 338,350 µg/L) (P=0.51) and 34,520 µg/L (range 3,794 - 325,400 µg/L) (P=0.19), during the first and second 2-hour incubations.

Conclusions—Retrieved human cerebral thromboemboli were amenable to ex vivo lysis by plasmin, the rate and degree of which was not different than that achieved with rt-PA.

Introduction
Thrombolytic treatment of ischemic stroke by recombinant tissue plasminogen activator (rt-PA) improves the overall outcome but is accompanied by a 10-fold higher incidence of symptomatic intracranial hemorrhage (1,2). The direct-acting thrombolytics offer the potential of effective yet safe therapy, and results in animal models (3,4) suggest that there is a considerable margin of hemostatic safety compared with rt-PA (5). According to the proposed mechanism of action, plasmin binds to and dissolves clot when delivered by catheter locally, and plasmin that escapes into the circulation is rapidly inactivated by antiplasmin, thereby avoiding remote bleeding at vascular injury sites (3,6). Plasmin has shown promise as a safe thrombolytic agent in the treatment of occluded hemodialysis shunts (7), and is undergoing clinical testing for lysis of acute peripheral arterial graft occlusions (NCT00418483). Locally delivered plasmin is effective in recanalizing acute middle cerebral artery (MCA) occlusion in a rabbit model (8,9), suggesting that the agent is useful in human ischemic stroke, and a clinical trial has been initiated for this indication (NCT01014975).

The recent development and application of retrieval devices for removing thromboemboli from the cerebral arteries of patients with acute ischemic stroke has allowed a special opportunity to perform further studies of the offending material (10). Our aims in this study are first, to determine whether retrieved cerebral thromboemboli are amenable to plasmin thrombolysis ex vivo and second, to determine whether the ex vivo efficacy of plasmin is different from that with rt-PA. Our null hypotheses are that the thromboemboli will be degraded by plasmin and that the extent of degradation will not be different than that achieved with rt-PA.

Methods
Processing of cerebral thromboemboli
Cerebral thromboemboli (29 fragments) were retrieved from the intracranial carotid artery system (Merci retriever®, Concentric Medical Inc., Mountain View, California) of 15 patients with acute ischemic stroke treated at the Ronald Reagan UCLA Medical Center, Los Angeles, California. Thromboemboli were numbered without reference to patient identity according to protocol approved by the UCLA IRB, and stored at −80°C until shipment on dry ice to the laboratory of A.B. (University of Ljubljana Medical Centre, Ljubljana, Slovenia). The 29 fragments were randomized for ex vivo exposure to plasmin or rt-PA (Table 1) by a randomly generated number (www.random.org).

Ex vivo thrombolysis
Plasmin (Human, TAL-05-00018) was kindly provided by Talecris Biotherapeutics (Research Triangle Park, North Carolina), dissolved with 0.9 % sodium chloride (pH 4.9) at 5 mg/ml, frozen at −80°C, and diluted for use at 1mg/ml with distilled water (pH 7.0). The rt-PA (Actilyse®, Boehringer Ingelheim GmbH, Ingelheim am Rhein, Germany) was diluted to 1mg/ml with 0.9 % Sodium chloride (pH 4.9) and aliquots frozen at −80°C. Type AB fresh frozen plasma was obtained from the Centre for Transfusion Medicine, Republic of Slovenia.
Thromboembolic fragments were gently blotted free of surface liquid, then suspended in 1 ml of plasma and gently stirred in a 37°C water bath (11). The rt-PA was added as 320 µl at time 0 (pH 7.19) and an additional 160 µl after 1 hour, and plasmin as 400 µl at time 0 (pH 7.36) and an additional 200 µl after 1 hour, for final concentrations of 3.6 µmol/L for one hour and 4.8 µmol/L for the second hour for both agents. After 2 hours, clots were removed, photographed on a millimeter grid for area measurement, and suspended in 1 ml of plasma containing the same sequence of the other agent for crossover exposure.

Venous blood (4.5 mL) of normal volunteers was obtained after informed consent, collected onto 0.45 mL of 0.129 mol/L sodium citrate (BD Biosciences, Belgium), aliquots (1.0 mL) were treated with 100 µl of thrombin (final concentration 10 units/mL) (Sigma Aldrich, Germany) and 50 µl of calcium chloride (20 mmol/L). Clots were allowed to retract for 8 hours at 25°C, expelled serum removed and clots stored at −70°C. Fragments were weighed on an electronic balance with an accuracy of 0.1 mg. Two-dimensional area was analyzed from photographs on a millimeter grid background. Surface area was measured by the ImageJ program (National Institutes of Health, Bethesda, Maryland, USA), in which color images were converted into 8-bit grey-scale, with pixel intensity of 0 corresponding to completely black and 255 to completely white (12). Surface area was adjusted for image intensity (I₀/I₂h and I₀/I₄h), and the adjusted surface area was expressed as S_adj(2h) = S × I₀/I₂h and S_adj(4h) = S × I₀/I₄h. During the course of experiments, we observed a strong correlation between initial weight and area of thromboemboli. As post-exposure sample weight could not be accurately measured, we have assumed that surface area alone is an adequate measure for determining change in sample size.

After 2 and 4 hours of exposure to agent, 100 µl of plasma was withdrawn into tubes containing 100 µL bovine pancreatic trypsin inhibitor (Aprotinin®, Bayer AG, Leverkusen, Germany) at 20,000 KIU/mL and frozen at −70°C. D-dimer was measured by the Biopool Auto-Dimer® turbidimetric latex agglutination method (TrinityBiotech, Lemco, Germany) using the BCT analyzer (Dade Behring, Siemens AG, Munich, Germany) (13). D-dimer concentration after 2 and 4 hours were used as the measure of fibrin degradation during the prior 2-hour incubations.

**Statistical analysis**

Data for initial weight and two-dimensional area are presented as means and standard deviations. The differences were evaluated by t-test and Chi-square analyses. The released D-dimer that was not normally distributed is presented as median and range, and the differences between groups were evaluated by the non-parametric 2-tailed Wilcoxon-Mann-Whitney test.

**Results**

**Whole blood clot studies to determine agent concentrations**

Retracted whole blood clots were exposed for 2 hours to plasmin or rt-PA (2.9 or 4.7 µmol/L final concentration) or saline. Clot lysis was measured by % weight reduction and by released D dimer (Figure 1). Saline and plasmin at 2.9 µmol/L concentration showed minimal clot lysis. Plasmin at 4.7 µmol/L induced a 31 +/− 15.6% weight reduction and D dimer release of 200 +/− 106 µg/L per mg clot weight. Equimolar rt-PA induced similar results, 21 +/− 12% weight reduction and 135 +/− 70 µg/L D dimer/mg clot weight (P=0.28 and 0.19, respectively). On this basis, *ex vivo* experiments with thromboemboli utilized plasmin and rt-PA at 4.7 µmol/L final concentrations.
Pre-treatment size of thromboembolic fragments exposed initially to plasmin or rt-PA

The 14 samples exposed initially to plasmin showed a non-significant trend toward larger weight (26.6 +/- 22.9 mg vs 13.1 +/- 7.6 mg, P=0.10) and area (18.9 +/- 17.2 mm$^2$ vs 10.1 +/- 6.0 mm$^2$, P=0.15) than the 15 samples exposed initially to rt-PA (Figure 2A). This trend was likely due to all 3 fragments retrieved from the terminal ICA being randomized to the plasmin-first group (Table 1). There was excellent correlation (R = 0.914) of fragment weight and area (Figure 2B), on which basis either could be applied to monitoring thrombolysis.

Ex vivo lysis of cerebral thromboemboli by plasmin and rt-PA

Change in size—Initial 2-hour exposures to rt-PA and plasmin induced equivalent decreases in area, from 10.1 +/- 6.0 to 5.9 +/- 4.3 mm$^2$ for rt-PA and from 18.9 +/- 17.2 to 9.8 +/- 9.4 mm$^2$ for plasmin (P=0.25 for residual area and P=0.27 for absolute decrease in area) (Figure 3). Crossover exposures of residual thrombus for exposure to the other agent showed further decreases, from 5.9 +/-4.3 to 2.1 +/- 2.9 mm$^2$ for plasmin and from 9.8 +/- 9.4 to 5.0 +/- 4.4 mm$^2$ for rt-PA (P=0.10 for residual area and P=0.71 for absolute decrease in area).

Considering that the mean starting area of the thromboemboli treated with plasmin first was (non-significantly) larger, the results were also analyzed by % of initial area of remaining material after each 2-hour incubation. The first 2-hour rt-PA exposure reduced thrombus area to 67.3 +/- 57.0% of initial size, compared with 54.1 +/- 29.4% by plasmin (P=0.52). Crossover treatment of plasmin-exposed fragments with rt-PA further reduced area to 31.6 +/- 26.9% of initial and crossover treatment of rt-PA exposed fragments with plasmin reduced the area to 30.1 +/- 51.5 % of initial (P=0.97) (Fig 4A). These data reflect similar rates of lysis by rt-PA and plasmin, calculated both as absolute and relative decreases of thrombus area.

To test whether the initial exposure of fragments to agent may have changed lysis response to a second exposure, we compared initial versus followup lysis rates in two ways. Based on % remaining material, neither plasmin nor rt-PA showed more (or less) change in area during their second incubation (P = 0.26 and 0.14, respectively). Based on each fragment’s final versus starting area, all four incubations showed the same slope of the linear regression line (Figure 4B). The data indicate that prior exposure of fragments to either agent did not affect subsequent lysis rates with a second exposure, to the same or the other agent.

D-dimer release—There was no difference in D-dimer released with plasmin or rt-PA after the initial exposure (median 108,180 μg/L; range 16,780 - 668,050 μg/L vs. median 151,990; range 9,870 - 338,350 μg/L; P=0.51) or crossover exposure (median 16,905 μg/L; range 240 - 403,085 μg/L vs. median 3,794 - 325,400 μg/L; P=0.19)

However, there was more absolute D-dimer release during the initial 2-hour exposure than during the crossover treatment for both agents (P=0.05 for plasmin, P=0.02 for rt-PA) (Figure 5A). This was explained by the smaller fragment size at the beginning of the crossover exposure (about 60% of initial area) (Figure 4A). There was a good correlation between fragment area and released D-dimer (R=0.745) (Figure 5B). When calculated as D-dimer release per mm$^2$ area of fragment, there was no difference between rt-PA and plasmin (P=0.12 for initial exposure; P = 0.33 for crossover exposure) or between first and followup incubations (P=0.33 for rt-PA, P=0.57 for plasmin), as further evidence that initial exposure to agent did not affect followup thrombolysis (Figure 5C).

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Discussion

Our results demonstrate that plasmin induced significant *ex vivo* lysis of thromboemboli retrieved from patients with acute ischemic stroke, and that plasmin and rt-PA exerted lytic effect that was not significantly different. As antiplasmins are present in pathologic arterial thrombi (14), plasmin lysis of 70% of thrombus (Figure 4A) was gratifying, and provides foundation for clinical testing of plasmin in patients with ischemic stroke (9).

Our expectation was that *ex vivo* lysis by rt-PA might be less than achieved with plasmin (15), as PA-induced thrombolysis depends upon plasminogen, which is limited in organized thrombi (16). That rt-PA-induced thrombolysis was equivalent to that with plasmin could be the result of several influences. First, we chose the lowest effective concentration of plasmin that induced thrombolysis of retracted whole blood clots (Figure 1), a concentration that barely exceeded that of $\alpha_2$-antiplasmin (1 $\mu$mol/L) (17) and $\alpha_2$-macroglobulin (3 $\mu$mol/L) (18). On the contrary, rt-PA concentration clearly exceeded PAI-1 inhibition, mimicked that attained by catheter delivery (19), and may have accumulated onto thrombi during lysis (20), negating any restriction to rt-PA effect caused by limited plasminogen content.

Our study design tried to avoid bias by randomly assigning samples to plasmin or rt-PA, and crossover ensured that every sample was exposed to both agents. Further, results were measured by two distinctly different parameters (area and D dimer release). However, several aspects of our approach deserve mention. First, a flow (rather than a static) system would have been preferable, since pressure-driven clot permeation delivers thrombolytic agent more efficiently than diffusion alone (21) and flow mechanically removes partly degraded clot fragments (22). However, the fragments were too fragile to withstand a perfusion system without damage and distortion of experimental results.

Second, as the samples were small and fragile, determination of weight after agent exposure would not only risk losing portions of sample, but would be artefactually distorted by swelling fluid. We showed that 2-dimensional area strongly correlated with weight (Figure 2B), and thereafter utilized area as the measure of sample “size”. While imperfect, this approach was confirmed by quantifying D-dimer release not only as a total amount, but also in relation to units of area (mm$^2$) (Figure 5C). This finding also minimized concern that larger fragment size and ICA origin (3 of 14) of plasmin-first samples were of greater or lesser susceptibility to lysis than smaller fragments (Figure 1A, Table 1).

Third, question could be raised as to whether structural change induced by first agent exposure influenced results with the followup (crossover) exposure. This clearly did not happen, as reflected by the same pre- to post-exposure area relationships for initial as for followup incubations (Figure 4B) and by the same D dimer release per unit area after initial or followup exposures (for both plasmin and rt-PA) (Figure 5C).

Last, histology was not established prior to agent exposure, being precluded by small sample size that would jeopardize data collection. Further, histologic heterogeneity of these thromboemboli (10) would not allow conclusions to be drawn regarding the structure of sample portions that were actually exposed to agent.

In summary, we have documented that plasmin induces substantial lysis of cerebral artery thromboemboli retrieved from patients with acute ischemic stroke. Under the test conditions utilized, plasmin was not different from rt-PA in lysing these pathologic specimens. The results suggest that efficacy can be reasonably expected in the ongoing Phase I clinical trial of plasmin in patients with acute ischemic stroke.
Acknowledgments

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Figure 1. Dose-finding study of plasmin and rt-PA using retracted whole blood clots. Top panel shows clot weight reduction and lower panel shows D dimer released. Incubation with plasmin and rt-PA at 4.7 μmol/L induced 31% and 21% reductions in clot weight, respectively (P=0.28); non-significantly different results for each agent were obtained for D dimer released (P=0.19).
Figure 2. Size comparison of thromboembolic fragments randomized to initial exposure to rt-PA or plasmin.

**Figure 2A: Initial weight and area.** Group 1 was exposed to rt-PA first, then to plasmin after crossover; Group 2 was exposed to plasmin first and to rt-PA after crossover. There was a non-significant trend for greater weight (26.6 +/- 22.9 mg vs 13.1 +/- 7.6 mg.)
P=0.10) and area (18.9 +/- 17.2 mm$^2$ vs 10.1 +/- 6.0 mm$^2$, P=0.15) of thrombi in treatment group 2.

Figure 2B: Correlation of initial thrombus weight and area. There was a strong correlation of initial weight with initial area of the thromboembolic fragments (correlation coefficient R=0.914, R$^2$ = 0.835).
Figure 3. Fragment area at the start, after initial exposures, and after crossover exposures to rt-PA and plasmin
Black bars show the area of thromboemboli before and after treatment with rt-PA for 2 hours, which were then exposed to plasmin for 2 hours (grey bar “AFTER 4 H”). Grey bars show the area of thromboemboli before and after treatment with plasmin for 2 hours, which were then exposed to rt-PA for 2 hours (black bar “AFTER 4 H”).
Figure 4. Fragment area before and after exposure to plasmin and rt-PA
Figure 4A: Remaining thrombus (% initial area) after initial and crossover exposures. The slope of area change for plasmin (solid lines) and for rt-PA (interrupted lines) are not different for their initial and followup exposures (P = 0.26 and 0.13, respectively).
Figure 4B: Comparison of fragment initial versus post-exposure areas. The same relationship of initial and final areas obtained for all exposures, initial or followup and plasmin or rt-PA.
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Figure 5. D-dimer released from cerebral thromboemboli with plasmin or rt-PA

Figure 5A. Absolute amounts of D dimer released from starting thromboemboli and from residual particulate material after 2 hours of thrombolysis. Individual data points noted as solid circles, mean values as open circles (+/- SD). There were no significant differences in D-dimer release between plasmin and rt-PA after the first 2 hours or after crossover exposure. D-dimer release was higher after initial treatment than after crossover treatment for both agents.

Figure 5B. Amount of released D dimer relative to area of fragments at the start of initial and crossover exposures. Excellent correlation (R=0.744) of D dimer release and initial fragment size (area) at the start of initial and crossover exposures, despite the 60% smaller size of fragments after first exposure to agent.

Figure 5C. Comparison of D dimer release per unit area of starting material by rt-PA or by plasmin during each exposure. No difference in D dimer release when calculated per unit area, between initial and crossover exposures or between rt-PA and plasmin thrombolysis.
Table 1
Cerebral artery source and prior exposure of thromboembolic fragments to therapeutic rt-PA.

<table>
<thead>
<tr>
<th>INITIAL AGENT (prior rt-PA exposure)</th>
<th>rt-PA</th>
<th>PLASMIN</th>
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</thead>
<tbody>
<tr>
<td>Cerebral artery source of thromboembolic specimen</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Middle cerebral artery</td>
<td>15 (2)</td>
<td>11 (1)</td>
</tr>
<tr>
<td>Terminal internal carotid artery</td>
<td>0</td>
<td>3</td>
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