Comparison of Plasmin With Recombinant Tissue-Type Plasminogen Activator in Lysis of Cerebral Thromboemboli Retrieved From Patients With Acute Ischemic Stroke

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Background and Purpose—Plasmin is a direct-acting thrombolytic with a better safety profile than recombinant tissue-type plasminogen activator (rtPA) 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 rtPA.

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 rtPA. 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 68.4% ± 26.9% and inducing median d-dimer release of 108 180 μg/L (range, 16 780 to 668 050 μg/L) and 151 990 μg/L (range, 240 to 403 085 μg/L) during the first and second 2-hour incubation periods, respectively. These changes were not different from those obtained with rtPA, 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 108 180 μg/L (range, 9870 to 338 350 μg/L; P = 0.51) and 34 520 μg/L (range 3794 to 668 050 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 rtPA. (Stroke. 2011;42:2222-2228.)

Key Words: embolic stroke ■ thrombolysis

Thrombolytic treatment of ischemic stroke by recombinant tissue-type plasminogen activator (rtPA) 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 models3,4 suggest that there is a considerable margin of hemostatic safety compared with rtPA.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 shunts7 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 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 rtPA. Our hypothesis is that the thromboemboli will be degraded by plasmin while the null hypothesis is that the extent of degradation will not be different than that achieved with rtPA.
Table. Cerebral Artery Source and Prior Exposure of Thromboembolic Fragments to Therapeutic rtPA

<table>
<thead>
<tr>
<th>Cerebral Artery Source of Thromboembolic Specimen</th>
<th>Initial Agent (Prior rtPA Exposure)</th>
<th>Plasmin</th>
<th>rtPA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Middle cerebral artery</td>
<td>rTPA</td>
<td>15 (2)</td>
<td>11 (1)</td>
</tr>
<tr>
<td>Terminal internal carotid artery</td>
<td>Plasmin</td>
<td>0</td>
<td>3</td>
</tr>
</tbody>
</table>

rtPA indicates recombinant tissue plasminogen activator.

Methods

Processing of Cerebral Thromboemboli

Cerebral thromboemboli (29 fragments) were retrieved from the intracranial carotid artery system (Merci retriever; Concentric Medical Inc, Mountain View, CA) of 15 patients with acute ischemic stroke treated at the Ronald Reagan University of California–Los Angeles Medical Center, Los Angeles, CA. Thromboemboli were numbered without reference to patient identity according to protocol approved by the University of California–Los Angeles Institutional Review Board 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 rtPA (Table) 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, NC), dissolved with 0.9% sodium chloride (pH 4.9) at 5 mg/mL, frozen at −80°C, and diluted for use at 1 mg/mL with distilled water (pH 7.0). The rtPA (Actilyse; Boehringer Ingelheim GmbH, Ingelheim am Rhein, Germany) was diluted to 1 mg/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.² The rtPA 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 1 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), and aliquots (1.0 mL) were treated with 100 μL of thrombin (final concentration 10 U/mL; Sigma Aldrich) 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. A 2-dimensional area was analyzed from photographs on a millimeter grid background. Surface area was measured by the ImageJ program (National Institutes of Health, Bethesda, MD), in which color images were converted into 8-bit gray-scale with pixel intensity of 0 corresponding to completely black and 255 to completely white.¹² Surface area was adjusted for image intensity (L(Ih1/hour) and L(Ih2/hour), and the adjusted surface area was expressed as $S_{adj1(2)} = S 	imes L(Ih1/Ih2)$. During the course of experiments, we observed a strong correlation between initial weight and area of thromboemboli. Because postexposure 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 2-dimensional area are presented as means and standard deviations. The differences were evaluated by t test and $\chi^2$ 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 nonparametric 2-tailed Wilcoxon Mann–Whitney U test.

Results

Whole Blood Clot Studies to Determine Agent Concentrations

Retracted whole blood clots were exposed for 2 hours to plasmin or rtPA (2.9 or 4.7 μmol/L final concentration) or saline. Clot lysis was measured by percent 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/mg clot weight. Equimolar rtPA induced similar results, 21%±12% weight reduction and 135±70 μg/L/d-mger/mg clot weight ($P=0.28$ and 0.19, respectively). On this basis, ex vivo experiments with thromboemboli used plasmin and rtPA at 4.7 μmol/L final concentrations.

Pretreatment Size of Thromboembolic Fragments Exposed Initially to Plasmin or rtPA

The 14 samples exposed initially to plasmin showed a nonsignificant trend toward larger weight (26.6±22.9 mg versus 13.1±7.6 mg, $P=0.10$) and area (18.9±17.2 mm² versus 10.1±6.0 mm², $P=0.15$) than the 15 samples exposed initially to rtPA (Figure 2A). This trend was likely due to all 3 fragments retrieved from the terminal internal carotid artery being randomized to the plasmin-first group (Table). 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 rtPA

Change in Size

Initial 2-hour exposures to rtPA and plasmin induced equivalent decreases in area, from 10.1±6.0 to 5.9±4.3 mm² for rtPA and from 18.9±17.2 to 9.8±9.4 mm² 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 mm² for plasmin and from 9.8±9.4 to 5.0±4.4 mm² for rtPA ($P=0.10$ 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 (nonsignificantly) larger, the results were also analyzed by percent of initial area of remaining material after each 2-hour incubation.
The first 2-hour rtPA exposure reduced thrombus area to $67.3\% \pm 57.0\%$ of initial size compared with $54.1\% \pm 29.4\%$ by plasmin ($P=0.52$). Crossover treatment of plasmin-exposed fragments with rtPA further reduced area to $31.6\% \pm 26.9\%$ of initial and crossover treatment of rtPA-exposed fragments with plasmin reduced the area to $30.1\% \pm 51.5\%$ of initial ($P=0.97$; Figure 4A). These data reflect similar rates of lysis by rtPA 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 follow-up lysis rates in 2 ways. Based on percent remaining material, neither plasmin nor rtPA showed more (or less) change in area during their second incubation \((P=0.26 \text{ and } 0.14, \text{ respectively})\). Based on each fragment’s final versus starting area, all 4 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 rtPA after the initial exposure (median, 108 180 µg/L; range, 16 780 to 668 050 µg/L versus median, 151 990 µg/L; range, 9870 to 338 350 µg/L; \(P=0.51\)) or crossover exposure (median, 16 905 µg/L; range, 240 to 403 085 µg/L versus...
median, 34 520 μg/L; range, 3794 to 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 rtPA; Figure 5A). This was explained by the smaller fragment size at the beginning of the crossover exposure (approximately 60% of the 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 square millimeter area of fragment, there was no difference between rtPA and plasmin (P=0.12 for initial exposure; P=0.33 for crossover exposure) or between first and follow-up incubations (P=0.33 for rtPA, P=0.57 for plasmin), because further evidence that initial exposure to agent did not affect follow-up thrombolysis (Figure 5C).

**Discussion**

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

Our expectation was that ex vivo lysis by rtPA might be less than achieved with plasmin, because PA-induced thrombolysis depends on plasminogen, which is limited in organized thrombi. That rtPA-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, rtPA concentration clearly exceeded plasminogen activator inhibitor-1 inhibition, mimicked that attained by catheter delivery,$^{19}$ and may have accumulated onto thrombi during lysis,$^{20}$ negating any restriction to rtPA effect caused by limited plasminogen content.

Our study design tried to avoid bias by randomly assigning samples to plasmin or rtPA, and crossover ensured that every sample was exposed to both agents. Furthermore, results were measured by 2 distinctly different parameters (area and $\alpha$-dimer release). However, several aspects of our approach deserve mention. First, a flow (rather than a static) system would have been preferable, because 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, because the samples were small and fragile, determination of weight after agent exposure would not only risk losing portions of sample but would be artifactually distorted by swelling fluid. We showed that 2-dimensional area strongly correlated with weight (Figure 2B) and thereafter used area as the measure of sample “size.” Although imperfect, this approach was confirmed by quantifying $\alpha$-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 internal carotid artery origin (3 of 14) of plasmin-first samples were of greater or lesser susceptibility to lysis than smaller fragments (Figure 1A; Table).

Third, a question could be raised as to whether structural change induced by first agent exposure influenced results with the follow-up (crossover) exposure. This clearly did not

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Figure 5. $\alpha$-dimer released from cerebral thromboemboli with plasmin or rtPA. A, Absolute amounts of $\alpha$-dimer released from starting thromboemboli and from residual particulate material after 2 hours of thrombolysis. Individual data points noted as solid circles and mean values as open circles (±SD). There were no significant differences in $\alpha$-dimer release between plasmin and rtPA after the first 2 hours or after crossover exposure. $\alpha$-dimer release was higher after initial treatment than after crossover treatment for both agents. B, Amount of released $\alpha$-dimer relative to area of fragments at the start of initial and crossover exposures. Excellent correlation ($R = 0.744$) of $\alpha$-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. C, Comparison of $\alpha$-dimer release per unit area of starting material by rtPA or by plasmin during each exposure. No difference in $\alpha$-dimer release when calculated per unit area, between initial and crossover exposures, or between rtPA and plasmin thrombolysis. rtPA indicates recombinant tissue-type plasminogen activator.
happen as reflected by the same pre- to postexposure area relationships for initial as for follow-up incubations (Figure 4B) and by the same D-dimer release per unit area after initial or follow-up exposures (for both plasmin and rtPA; Figure 5C).

Last, histology was not established before agent exposure, being precluded by small sample size that would jeopardize data collection. Furthermore, histological heterogeneity of these thromboemboli 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 used, plasmin was not different from rtPA in lysing these pathological 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.

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