Comparative Effects of Tissue Plasminogen Activator, Streptokinase, and Staphylokinase on Cerebral Ischemic Infarction and Pulmonary Clot Lysis in Hamster Models

N. Nagai, PhD; I. Vanlinthout; D. Collen, MD, PhD

**Background**—The effects of alteplase (rtPA), streptokinase, and staphylokinase (rSak) on focal cerebral ischemia (FCI) and on pulmonary clot lysis (PCL) were studied in hamsters.

**Methods and Results**—FCI was produced by ligation of the left middle cerebral artery (MCA) and common carotid artery (CCA) and a 10-minute occlusion of the right CCA. FCI was measured after 24 hours by 2,3,5-triphenyltetrazolium chloride staining. Infusion of rtPA, streptokinase, and staphylokinase (rSak) in hamsters, have a sensitivity for clot lysis with rtPA and streptokinase, and rSak on FCI and on pulmonary clot lysis (PCL) were studied in hamsters. Hamsters, in contrast to mice, have a sensitivity for clot lysis with rtPA and streptokinase. Wang et al. recently demonstrated that rtPA increased the volume of FCI after occlusion of the middle cerebral artery (MCA) in mice. The hypothesis was that focal ischemia, via oxidative stress, released excitotoxins that sensitize neurons to cell death and that tPA increases neuronal cell death. In the hippocampus, the effect of tPA occurs via plasmin generation, which degrades laminin and disrupts the interaction between neurons and extracellular matrix, resulting in enhanced neuronal cell death. These observations raise several questions on the relationship between thrombolytic therapy and FCI: (1) Is it a species-specific or general phenomenon? (2) Is it agent-specific or a class feature of thrombolytic agents? and (3) Is it related to systemic plasmin generation?

**Conclusions**—Thrombolytic agents cause similar dose-related extension of FCI after MCA ligation and PCL, irrespective of the agent or systemic plasmin generation. (Circulation. 1999;100:2541-2546.)

**Key Words:** cerebral infarction ■ plasminogen activators ■ streptokinase ■ stroke ■ thrombolysis

Thrombolytic therapy of ischemic stroke with alteplase (recombinant tissue plasminogen activator, rtPA) improved clinical outcome at 3 months, whereas trials with streptokinase were terminated prematurely because of excess mortality. Infusion of rtPA or staphylokinase (rSak) in rabbits with embolic stroke reduced focal cerebral ischemic infarction (FCI) and neurological deficit. To date, however, rSak has not been given to patients with ischemic stroke. Wang et al. recently demonstrated that rtPA increased the volume of FCI after occlusion of the middle cerebral artery (MCA) in mice. The hypothesis was that focal ischemia, via oxidative stress, released excitotoxins that sensitize neurons to cell death and that tPA increases neuronal cell death. In the hippocampus, the effect of tPA occurs via plasmin generation, which degrades laminin and disrupts the interaction between neurons and extracellular matrix, resulting in enhanced neuronal cell death. These observations raise several questions on the relationship between thrombolytic therapy and FCI: (1) Is it a species-specific or general phenomenon? (2) Is it agent-specific or a class feature of thrombolytic agents? and (3) Is it related to systemic plasmin generation?

In the present study, the comparative effects of rtPA, streptokinase, and rSak on FCI and on pulmonary clot lysis (PCL) were studied in hamsters. Hamsters, in contrast to mice, have a sensitivity for clot lysis with rtPA and streptokinase, similar to that of humans. Furthermore, it is possible to “humanize” the thrombolytic response in terms of systemic plasmin generation and fibrinogen breakdown by infusion of human plasminogen. The present results confirm that thrombolytic agents increase FCI size after MCA ligation; this phenomenon, however, is neither species-specific nor agent-specific, and it occurs in the absence of systemic plasmin generation and fibrinogen breakdown.

**Methods**

**Study Drugs**
The rtPA (alteplase) used was Actilyse, a gift from Boehringer Ingelheim, Ingelheim, Germany. Streptokinase was Streptase, obtained from Hoechst, Brussels, Belgium, and recombinant staphylokinase (rSak, natural variant SakSTAR) was produced, purified, and characterized as described elsewhere.

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Hamster Cerebral Ischemic Infarction Model
Animal experiments were conducted according to the guiding principles of the International Committee on Thrombosis and Hemostasis.\textsuperscript{14} FCI was produced by persistent occlusion of the left MCA and common carotid artery (CCA) and a transient occlusion of the right CCA. Hamsters (Pfd Gold, University of Leuven, Belgium) were anesthetized with ketamine 75 mg/mL IP (Apharmo) and xylazine 5 mg/mL IP (Bayer). Atropine 1 mg/kg IM (Federa) was administered, and body temperature was monitored. A reversed U-shape incision was made between the left ear and left eye. The top, forward, and bottom segments of the temporal muscle were transected and retracted. A small opening (2 to 3 mm in diameter) was made in the region of the MCA with a handheld drill, with saline superfusion to prevent heat injury. The inner layer of the skull and the meninges were removed with a forceps, the MCA was ligated with 10-0 nylon thread (Ethylon), and the artery was transected distally. Finally, the temporal muscle and skin were sutured back in place.

The animals were then placed in the supine position, and both CCAs were exposed. The left CCA was ligated with two 7-0 nylon threads and transected between them. The right CCA was occluded with an arterial clamp for 0, 10, or 30 minutes, respectively, in groups of 6 animals to determine optimal conditions for the induction of FCI. For subsequent experiments, a 10-minute occlusion time was used. Finally, the skin wound was closed, and the femoral vein was cannulated with a 2F catheter for study drug administration.

Groups of \( \geq 6 \) hamsters were randomly allocated to rtPA, streptokinase, or rSak, but when the animals remained unresponsive after recovery from anesthesia (usually associated with bilateral FCI and/or extensive cerebral edema), they were replaced with additional experimental animals to restore the group size. The groups with their dose ranges, number of animals, and exclusions are represented in Table 1 under Cerebrovascular Parameters. In total, 104 experiments were performed, of which 96 were analyzable. The study drugs were infused over a period of 60 minutes. After 24 hours, the animals were euthanized with an overdose of pentobarbital sodium 500 mg/kg (Nembutal, Abbott) and decapitated. The brain was removed, sectioned into 1-mm segments, immersed in 2% 2,3,5-triphenyltetrazolium chloride (TTC) in saline for 30 minutes at 37°C, placed in 4% formalin in PBS, and photographed. Thus, the necrotic infarct area remains unstained (white) and is clearly distinguishable from stained (brick red) viable tissue (Figure 1). Independently, the photographs were subjected to planimetry, and infarct volume was defined as the sum of the unstained areas of the sections multiplied by their thickness.

### Table 1. Comparative Effects of rtPA, Streptokinase, and rSak on FCI and on PCL in Hamsters

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Cerebrovascular Parameters (at 24 h)</th>
<th>Clot Lysis Parameters (at 90 min)</th>
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<tbody>
<tr>
<td>Compound</td>
<td>Dose, ( \mu g/kg )</td>
<td>Plasminogen, ( mg/kg )</td>
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<td>Without Human Plasminogen</td>
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<td>rSak</td>
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The cerebral infarct data represent median and 17–83 percentile range of \( N \)–\( n \) experiments. The clot lysis data represent mean±SEM of \( N \) experiments.
The effect of systemic plasminogen activation on FCI was studied by injection of 3 or 6 mg human plasminogen/kg body wt, followed by infusion of 27, 81, or 240 μg/kg streptokinase in groups of 6 analyzable animals. In total, 29 experiments were carried out, of which 24 were analyzable (Table 1, section Cerebrovascular Parameters). FCI was then measured as described above.

Hamster Pulmonary Embolism Model

125I-fibrin–labeled hamster plasma clots (50 μL) were injected into the jugular vein of heparinized hamsters, as described previously. rtPA, streptokinase, rSak, or saline was infused over 60 minutes, and clot lysis was quantified at 90 minutes as the difference between the radioactivity incorporated into the clot and the residual radioactivity in the lungs and the heart. The group size was ≥4 animals per dose (total 71 experiments), as detailed in Table 1, section Clot Lysis Parameters.

Blood samples (0.2 mL) were drawn into trisodium citrate (0.011 mol/L, final concentration) for measurement of radioactivity, fibrinogen, and α2-antiplasmin. An isotope recovery balance was made by adding the radioisotope content recovered in the heart and lungs and in the blood (the latter multiplied by a factor of 3 to correct for the extravascular distribution space of free iodide and iodopeptides) at the end of the experiment.

A bolus injection of 3 or 6 mg human plasminogen/kg body wt was given to study the effect of systemic plasminogen activation on clot lysis (Figure 1). In each group, 27, 81, or 240 μg/kg of streptokinase or rSak (total of 40 experiments), as detailed in Table 1, section Clot Lysis Parameters. PCL and hemostatic parameters were then determined as described above.

Analysis of Data

Thrombolytic potency and effect on infarct expansion were determined as follows. The values of FCI or of PCL versus dose of thrombolytic agent were graphically fitted with an exponentially transformed sigmoidal function,

\[ y = \frac{Mc}{1 + e^{-(x-a)e^{-b}}}, \]

by use of the statistical program Grafit (Erithacus Ltd). In this equation, y represents FCI (in mm³) or PCL (in percent), respectively; x represents the dose of thrombolytic agent (in mg/kg); M is a constant that was set at 30 for the maximal FCI and at 100 for the maximal PCL; c is maximal fractional FCI or PCL achieved; b is the dose of thrombolytic agent (in mg/kg) at which the rate (slope of the dose-response curve) of FCI or PCL is maximal; and z (\(c^2a^3\)) is the maximal rate of FCI (in mm³·mg⁻¹·kg compound infused⁻¹) or maximal rate of PCL (in percent lysis · mg⁻¹ · kg compound infused⁻¹). These parameters were obtained as mean ± SEM, and the significance of the differences between these parameters was determined with Student’s t test for normally distributed values or with Welch’s test otherwise.

Results

Development of the FCI Model

Ligation of both the left MCA and CCA did not consistently produce FCI, as revealed by TTC staining, whereas additional persistent occlusion of the right CCA produced extensive cerebral necrosis and death of the animals (data not shown).

Therefore, persistent occlusion of the left MCA and CCA was combined with transient occlusion of the right CCA. FCI size was 1.3 (1.1 to 1.7) mm³ (median and 17th to 83rd percentile range, n=6) without occlusion of the right CCA, 1.4 (1.0 to 3.8) mm³ with 10-minute occlusion, and 28 (20 to 47) mm³ with 30-minute occlusion. In 3 animals in the group with 30-minute occlusion, extensive bilateral FCI and unresponsiveness were observed. Therefore, persistent occlusion of the left MCA and CCA combined with 10-minute occlusion of the right CCA was selected for subsequent experiments.

Comparative Effects of rtPA, Streptokinase, and rSak on FCI

Intravenous rtPA, streptokinase, or rSak produced dose-dependent increases of FCI (Table 1). In the rtPA groups, FCI was 0.7 (0.33 to 1.1) mm³ with solvent (data not shown separately but rather for all control groups combined in Table 1) and increased to 15 (12 to 22) mm³ with 500 μg/kg rtPA. In the streptokinase groups, FCI increased from 0.97 (0.92 to 2.1) mm³ with saline and increased to 30 (3.3 to 38) mm³ with 720 μg/kg streptokinase. In the rSak groups, FCI increased from 0.76 (0.92 to 2.1) mm³ with saline and increased to 30 (3.3 to 38) mm³ with 240 μg/kg .

Fitting of the dose-response data (Figure 2, left) yielded values for Mc (maximal FCI achieved), b (dose at which maximal rate of FCI occurs), and z (maximal rate of FCI), as summarized in Table 2. The effect of the agents on FCI, which is proportional to z and inversely proportional to b,
expressed in molar quantities ($M$, of rtPA, streptokinase, and rSak, 70 000, 43 000, and 16 500, respectively), was not significantly different among agents. The doses of compound at which half-maximal brain infarction or clot lysis is observed (obtained by graphical interpolation of Figure 2). The data represent mean±SEM, derived from the data in Table 1.

**Effect of Systemic Plasminemia on Cerebral Infarct Size and PCL**

Systemic plasminemia, characterized by $\alpha_2$-antiplasmin consumption and fibrinogen degradation to $<50\%$ of baseline, was obtained with high-dose streptokinase ($240 \mu g/kg$) preceded by injection of human plasminogen at a dose of 3 or 6 mg/kg (Table 1). This resulted in FCIs of 12 (3.8 to 26) and 17 (2.3 to 35) mm$^3$, respectively, which were not significantly different from 5.4 (4.3 to 6.3) mm$^3$ without human plasminogen and from 30 (3.3 to 38) mm$^3$ with 720 $\mu g/kg$ streptokinase alone. Fitting of all dose-response data obtained with streptokinase and human plasminogen (Figure 2, bottom left) yielded values for $b$ that were 2.5-fold lower and for $z$ that were 2-fold higher than obtained with streptokinase alone. These findings are suggestive of a larger effect of the combination, but these differences were not statistically significant ($P=0.26$ for $b$ value).

PCL with the combination of 240 $\mu g/kg$ streptokinase preceded by 3 or 6 mg/kg human plasminogen (Table 1) resulted in $83\%$ and $90\%$ lysis, which is significantly higher than $51\%\pm7\%$ with $240 \mu g/kg$ streptokinase without human plasminogen but comparable to $81\%\pm7\%$ with 720 $\mu g/kg$ streptokinase alone. Fitting of all dose-response data obtained with streptokinase and human plasminogen (Figure 2, bottom right) yielded values for $b$ that were 15-fold lower and for $z$ that were 25-fold higher ($P=0.006$ versus strept-
torkinase alone) than with streptokinase alone. These findings are indicative of a significantly higher thrombolytic potency of the combination versus streptokinase alone.

As expected, the combination of 250 μg/kg rSak and 6 mg/kg human plasminogen did not induce systemic plasminogen generation (Table 1). Therefore, the effect of this combination on FCI expansion was not studied.

**Discussion**

The present study was triggered by the recent observations by Wang et al7 that rtPA infusion increased infarct volume after FCI induced by guidewire obstruction of the MCA in mice. These findings suggested that in the presence of persisting cerebral arterial occlusion, rtPA administration might provoke toxic effects that counteract its beneficial thrombolytic effect on ischemic stroke, but they left open several clinically important questions. Here, some of these questions (species-specificity, agent-specificity, ratio of thrombolytic effect versus FCI expansion of several agents, and effect of fibrin-specificity on FCI generation) are addressed. Therefore, several choices had to be made, including the agents to be compared, the species to be tested, and procedures to produce fibrin-selective and non–fibrin-selective clot lysis.

rtPA and streptokinase are the most commonly used thrombolytic agents to treat acute myocardial infarction.15 rtPA is superior to streptokinase for coronary artery recanalization and reduction of mortality, probably because of the differential fibrin-selectivity of these agents.16,17 Streptokinase indeed represents the archetype of a non–fibrin-selective agent that in humans produces thrombus dissolution only in association with fibrinogen depletion, whereas rtPA represents the archetype of a (partially) fibrin-selective agent that at therapeutic doses will on average reduce the fibrinogen concentration only to two thirds of baseline. In ischemic stroke, rtPA has been found to improve clinical outcome,12 whereas streptokinase was associated with increased mortality.3–5 necessitating premature termination of several clinical trials with the latter agent. The intriguing question therefore arises of whether the difference in clinical efficacy between these drugs relates to their difference in thrombolytic potency or to their relative propensity for systemic plasminogen generation.

If animal models were available that would react comparably to humans, this hypothesis could be verified experimentally. In addition, the contribution of systemic plasminemia to FCI expansion could be further tested with the uniquely fibrin-selective staphylokinase (rSak).10,11 The murine species is unsuitable for this purpose, because it is refractory to clot lysis with streptokinase and with rSak (unpublished observations). Hamsters, however, react to rtPA, streptokinase, and rSak, in a PCL model, in proportion to their therapeutic doses in humans.

In the hamster, ligation of the left MCA, or even of both the left MCA and the left CCA, did not induce FCI. Additional transient occlusion of the right CCA was necessary to obtain FCI, with corresponding infarct volumes of 1.4 mm³ with 10-minute occlusion and 28 mm³ with 30-minute occlusion. Because the latter was frequently associated with bilateral cerebral necrosis and clinical deterioration, a right CCA occlusion time of 10 minutes was used for experiments with study drugs.

rtPA, streptokinase, and rSak produced dose-related lysis of PCL with comparable half-maximal values at 0.18, 0.23, and 0.021 mg/kg, respectively, and dose-related FCI with half-maximal volumes at 0.13, 0.27, and 0.038 mg/kg, respectively, without systemic α₂-antiplasmin consumption and fibrinogen breakdown. Thus, the results indicate that (1) FCI expansion with rtPA, previously demonstrated in the mouse,7 also occurs in another species and therefore probably in humans; (2) the effect is not agent-specific; (3) the effect on FCI occurs in the absence of systemic plasminogen generation, and (4) the dose-effect curve for FCI expansion roughly parallels that of PCL, with half-maximal and maximal values occurring at similar concentrations.

In an effort to define the contribution of systemic plasminogen generation to PCL and FCI expansion, the hamster fibrinolytic system was humanized by injection of purified human plasminogen. In humans, the plasminogen concentration in plasma is 200 mg/L and its half-life 2 days.18 A bolus injection of 3 or 6 mg/kg would thus raise the level of human plasminogen in hamster plasma to nearly physiological levels during the 60-minute infusion of study drugs. As expected, no systemic activation was obtained with rSak, which is highly fibrin-selective in humans, whereas extensive systemic plasminogen activation, characterized by α₂-antiplasmin consumption and fibrinogen breakdown to <50% of baseline, was obtained with 240 μg/kg streptokinase. Maximal rates of FCI were 2-fold higher and occurred at a 2.5-fold lower dose of streptokinase with versus without plasminogen, but these differences were not statistically significant. With the combination of streptokinase and human plasminogen, however, a 10-fold higher dose was necessary to obtain half-maximal FCI than required for half-maximal PCL.

PCL with streptokinase was significantly enhanced by administration of human plasminogen. This increased potency, however, might be due to either an intrinsic higher fibrinolytic activity of human plasmin, a greater sensitivity of human plasminogen to activation by streptokinase, or a disturbance of the relative concentrations of plasminogen and α₂-antiplasmin.

One shortcoming of the hamster FCI model is that hemorrhagic conversion of ischemic stroke or thrombolysis-associated intracranial hemorrhage was not observed, which precluded evaluation of their contribution to cerebrovascular accidents in association with thrombolytic therapy.

Thrombolytic therapy for ischemic stroke is based on the premise that timely recanalization of the occluded cerebral artery may salvage the “ischemic penumbra,”19 the hypoperfused but potentially viable zone adjacent to the central ischemic area; limit infarct size; and improve functional recovery and survival. Early thrombolysis with rtPA indeed restored reperfusion, salvaged jeopardized brain tissue, and limited FCI size in experimental animals4 and reduced morbidity and mortality in patients.1,2 The results of the study by Wang et al7 and of the present study, however, indicate that at least in patients with persistent occlusion, there may be a detrimental side effect of the thrombolytic agent, causing infarct expansion. Extrapolation of findings to thrombolytic
therapy of ischemic stroke would suggest that the beneficial clinical outcome with rtPA versus the harmful outcome with streptokinase might relate primarily to differences in efficacy for beneficial arterial recanalization (which is higher with rtPA). Because it appears to be impossible to distinguish a priori between patients who will and those who will not achieve cerebral arterial recanalization with rtPA, the development of specific conjunctive strategies to counteract its effects on persisting FCI appear to be warranted. In view of the interactive effects of oxidative stress and excitotoxin induction with thrombolytic agents on neuronal degeneration, oxygen radical scavengers, glutamate antagonists, or both might beneficially affect the clinical outcome of thrombolytic therapy for ischemic stroke.

Finally, the mechanism of infarct expansion with thrombolytic agents in the presence of persistent arterial occlusion remains enigmatic. Plasmin-mediated laminin degradation may play a role in hippocampal neuronal cell death but does not explain cortical neuronal cell death, which is increased in mice with plasminogen deficiency and reduced in mice with α2-antiplasmin deficiency. Direct, non-plasmin-mediated effects of plasminogen activators must be invoked to explain the observed FCI expansion, because direct plasmin infusion reduces FCI (Nagai et al, unpublished observations).

References

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