Thrombolytic Properties of Staphylokinase

By Osamu Matsuo, Kiyotaka Okada, Hideharu Fukao, Yoshiki Tomioka, Shigeru Ueshima, Masaaki Watanuki, and Masashi Sakai

We evaluated the properties of recombinant staphylokinase in comparison with those of tissue-type plasminogen activator (t-PA) and streptokinase (SK). The presence of fibrinogen fragment FCB-2 in the reaction mixture increased plasminogen activation by staphylokinase more than 20-fold. Such characteristics are similar to those of t-PA. On the other hand, SK was not affected by the presence of FCB-2. The thrombolytic properties of staphylokinase were studied in a system consisting of a radioactive human plasma clot suspended in the circulating citrated plasma. Significant thrombolysis (50% in 3 hours) was obtained with 2 μg/mL of staphylokinase and 4.45 μg/mL t-PA, as compared with 12 μg/mL for SK. The relative molar potency of staphylokinase, calculated from the molecular weight, was about two times more effective than that of SK, but about half of that of t-PA. Systemic fibrinolytic activation and fibrinogen breakdown was not observed with staphylokinase or t-PA, but was observed with SK. The thrombolytic efficiency of staphylokinase, which was calculated as the ratio of the degree of thrombolysis/the degree of fibrinogenolysis, was about five times greater than that of SK, and about half of that of t-PA. These findings suggest that staphylokinase has higher specific thrombolytic properties and lesser fibrinogenolytic properties than those of SK.

© 1990 by The American Society of Hematology.

WITH THE APPLICATION OF tissue-type plasminogen activator (t-PA), single-chain urokinase-type plasminogen activator (scu-PA), and anisolysed plasminogen-streptokinase activator complex (APSAC) to patients with acute myocardial infarction,123 thrombolytic therapy with plasminogen activators has entered a new era. Both t-PA and scu-PA digest fibrin predominantly without associated systemic fibrinogen breakdown. The first generation of plasminogen activators such as urokinase-type plasminogen activator (u-PA, UK) and streptokinase (SK) induce systemic plasminogen activation and marked fibrinogen breakdown, the so-called lytic state.45 However, as more patients underwent thrombolytic therapy with t-PA,7 it became apparent from results obtained in experimental animal models that the extent of fibrinogen breakdown was much greater than anticipated, although it was much smaller than that induced by SK. APSAC, which is a chemically modified plasminogen SK activator complex, can be administered by intravenous (IV) bolus administration, in contrast to the prolonged infusion required with IV SK. APSAC exhibited thrombolysis with negligible systemic activation of plasminogen and no fibrinogen breakdown in rabbits with experimental pulmonary emboli.9 However, in clinical trials APSAC digested plasma fibrinogen to a comparable extent, as SK and bleeding episodes were equally or more frequent after APSAC.10 Thus, t-PA, and to a greater extent APSAC, do not meet the criteria of absolute fibrin-specificity of an ideal thrombolytic agent. Early assessment of scu-PA has also shown that it has fibrin-specificity in patients with acute myocardial infarction.11

The fibrinolytic properties of staphylokinase, which is an extracellular protein produced by Staphylococcus aureus, have been known for a long time.1214 It is now understood that staphylokinase mediates clot lysis by forming a complex with plasminogen, in a manner analogous to that of SK. Purification of staphylokinase has been achieved by affinity chromatography on immobilized plasminogen.15 Recently, the staphylokinase gene of S aureus was cloned and expressed in Escherichia coli, and large amounts of purified material became available.16 The present study deals with the clot-dissolving properties of staphylokinase in an in vitro system. These results indicate that staphylokinase is a potent fibrin specific thrombolytic agent that offers promise for potential clinical application.

MATERIALS AND METHODS

Materials. Staphylokinase was produced in a transformed E coli and purified by ammonium sulfate precipitation and ion-exchange chromatography according to the method reported by Sakai.17 The purity of staphylokinase was more than 99% as confirmed by high performance liquid chromatography and electrophoresis methods. The specific activity of staphylokinase was 3.3 x 10^6 U/mg protein, with one unit being defined as a lysis area of 10 mm in diameter on the plasminogen-rich fibrin film. SK (Kabi AB, Stockholm, Sweden) was purified by ammonium sulfate precipitation and affinity chromatography with a Blue-Sepharose CL-6B column (Pharmacia, Uppsala, Sweden). The specific activity, determined by the same method as for staphylokinase, was 1.4 x 10^6 U/mg protein. t-PA was a recombinant product of the two-chain type (specific activity was 450,000 IU/mg protein) (Sumitomo Pharmaceutical Co Ltd, Osaka, Japan). Human fibrinogen was prepared according to the method of Blomback and Blomback18 and purified by lysine-Sepharose affinity chromatography. Labeling of fibrinogen with 125I was performed according to McFarlane.9 FCB-2 was produced by the method of Nieuwenhuizen et al.19 CBS 33.08 (H-D-Nleu-CHA-Arg-pNA) and S-2251 (H-D-Val-L-Leu-L-Lys-pNA), which are chromogenic substrates for plasmin, were purchased from Diagnostica Stago (Asnieres-sur-Seine, France) or Kabi Vitrum AB, respectively. Plasmin was purchased from Kabi AB.

Kinetic analysis. Kinetic parameters for plasminogen activation with staphylokinase (4.3 μg/mL), SK (12.5 μg/mL), or t-PA (20

From the Department of Physiology, Kinki University School of Medicine, Osaka, Japan.

Address reprint requests to Osamu Matsuo, MD, Department of Physiology, Kinki University School of Medicine, Osaka, Japan. Submitted November 1, 1989; accepted May 1, 1990.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1990 by The American Society of Hematology.

Blood, Vol 76, No 5 (September 1), 1990: pp 925-929

925
μg/mL) in the presence or absence of FCB-2 using S-2251 (1 mmol/L) as a substrate were obtained from the Lineweaver Burk plot, as Hoylaerts et al reported previously.21

Stability of staphylokinase in plasma. Staphylokinase or SK (final concentration, 600 mmol/L) was incubated in human plasma at 37°C, and plasma samples were taken at every hour. A globulin fraction of plasma was produced as previously described,22 and the lysis area on the fibrin plate and the fibrin-containing gel after the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)23 was measured.

Measurement of thrombolysis in a circulating plasma system in vitro. The thrombolytic properties of staphylokinase were compared with those of SK or t-PA in a circulating plasma system consisting of a radioactive human plasma clot suspended in circulating human plasma.9 Briefly, the radioactive plasma clot was produced in a test tube (75 mm in length, 10 mm in internal diameter) by reacting 1 mL of citrated human plasma with 125I-labeled fibrinogen (7.0 × 10⁶ cpm), 100 μL of 25 mmol/L CaCl₂, and 50 μL of human thrombin (final concentration, 5 NIH U/mL), and left for 10 minutes at room temperature. The resulting thrombus was placed into a Petri dish and washed with saline. The amount of 125I in the thrombus was subsequently measured with a gamma-counter. The radioactive thrombus was then transferred to a mesh bag in the circulating plasma system, which consisted of two chambers connected with tubes through a pump. In this system, 21 mL of fresh frozen plasma (pH = 7.64 ± 0.01), divided approximately equally between the clot containing chamber and reservoir, was kept at 37°C and was circulated with a pump at a flow rate of 1.95 mL/min.

Activator solution (staphylokinase, SK, or t-PA) was diluted with 50 mmol/L Tris-HCl buffer, pH 7.4, to give a volume of 6 mL at a designated concentration. A 1-mL aliquot was injected into the reservoir, and the remaining 5-mL aliquot was continuously infused into the reservoir of SK, or t-PA in a circulating plasma system at a flow rate of 0.04 mL/min. Plasma samples were taken before the injection of the activator and at 1-hour intervals during the experimental period. The degree of clot lysis was estimated from the radioactivity released into a Petri dish and washed with saline. The amount of 125I in the thrombus was subsequently measured with a gamma-counter. The radioactive thrombus was then transferred to a mesh bag in the circulating plasma system, which consisted of two chambers connected with tubes through a pump. In this system, 21 mL of fresh frozen plasma (pH = 7.64 ± 0.01), divided approximately equally between the clot containing chamber and reservoir, was kept at 37°C and was circulated with a pump at a flow rate of 1.95 mL/min.

Activator solution (staphylokinase, SK, or t-PA) was diluted with 50 mmol/L Tris-HCl buffer, pH 7.4, to give a volume of 6 mL at a designated concentration. A 1-mL aliquot was injected into the reservoir, and the remaining 5-mL aliquot was continuously infused into the reservoir of SK, or t-PA in a circulating plasma system at a flow rate of 0.04 mL/min. Plasma samples were taken before the injection of the activator and at 1-hour intervals during the experimental period. The degree of clot lysis was estimated from the radioactivity released and expressed as a percentage of the original clot. The total radioactivity recovered from the remaining plasma clot and circulating plasma was expressed as a percentage of the radioactivity in the original clot. The plasma samples were subsequently subjected to measurement of their fibrinogen and α₂-plasmin inhibitor.

Measurement of fibrinogen and α₂-plasmin inhibitor concentration. To assess the extent of plasminogen activation and fibrinogen breakdown, the fibrinogen and α₂-plasmin inhibitor in the circulating plasma were measured.

The concentration of fibrinogen in the plasma was determined by performing the plasma clotting rate assay of Claas,14 adapted to a microplate assay as described below. The present method is, in principle, an endpoint assay that measures the turbidity produced on fibrin formation. Briefly, the plasma samples were diluted five times with 25 mmol/L Veronal buffer, pH 7.75, containing 50 mmol/L of CaCl₂, and 50-μL aliquots were taken into duplicate wells of a microtiter plate. Then 22 μL of plasmin solution (0.20 μmol/L) (the specific activity; 20 U/mg protein) was added. The mixture was incubated at 37°C for 15 minutes, and 10-μL aliquots of CBS 33.08 solution (0.33 mmol/L) were added subsequently. After incubation at 37°C for 15 minutes, 180 μL of 2% citrate solution was added to each of the wells, and the changes in absorbance at 405 nm were measured.

RESULTS

Basic properties of staphylokinase. Effect of FCB-2 addition on plasminogen activation with staphylokinase was measured and compared with that in the absence of FCB-2. Plasminogen activation rate was very slow in the absence of FCB-2 (kcat 0.0013 s⁻¹), but increased remarkably in the presence of FCB-2 (kcat 0.0238 s⁻¹), bringing about a 30-times increase in kcat/Km. On the other hand, SK was not affected by the addition of FCB-2, and no changes in the kcat/Km ratio was observed. t-PA, which has high affinity for fibrin, increased kcat 0.0081 s⁻¹ to 0.0124 s⁻¹ after FCB-2 addition, increasing the value of kcat/Km more than 50 times.

The stability of staphylokinase and SK was examined in human plasma at 37°C, and the remaining activity was measured by the electrophoretic enzymography and the fibrin film method. After 5 hours of incubation, the remaining activity of staphylokinase was about 92% of the initial activity, as measured by enzymography, and about 84% as measured by the fibrin film method. Conversely, the remaining activity of SK after 5 hours of incubation was 32% as measured by enzymography and 37% as measured by the fibrin film method.

Thrombolytic activity of staphylokinase and SK in the artificial circulating system. The degree of thrombolysis induced by different amounts of staphylokinase and SK in the artificial circulating system containing 125I-labeled plasma clot is shown in Fig 1. Generally, the degree of thrombolysis with staphylokinase was much greater than that with SK. For example, 50% thrombolysis in 3 hours required 2 μg/mL of staphylokinase and 12 μg/mL of SK, as determined by interpolation. Thrombolysis of more than 50% was observed at staphylokinase concentrations of 1.25 μg or more per milliliter of plasma. On the other hand, thrombolysis of more than 50% was obtained at an SK concentration of around 10 μg/mL. Thus, the thrombolytic effect of staphylokinase in this system was about six times higher than that of SK. Under the same experimental conditions, 4.45 μg/mL of t-PA induced 50% lysis at 3 hours (data not shown). After correction for difference in molecular weight between staphylokinase and SK, staphylokinase was found to induce thrombolysis about two times more effectively than SK.

Systemic fibrinolytic activation. The extent of fibrinogen breakdown induced with staphylokinase or SK in the circulating plasma is shown in Fig 2A. Fibrinogen breakdown was negligible with staphylokinase within 6 hours at a concentration below 2.5 μg/mL. Conversely, SK induced 100% fibrinogenolysis at a concentration of 2.5 μg/mL, which induced only 20% thrombolysis. The thrombolytic activity of staphylokinase was shown (Fig 3A) as the degree of thrombolysis versus the degree of fibrinogenolysis. Staphylokinase at concentration below 2.5 μg/mL induced marked...
thrombolysis without an associated fibrinogen degradation. Staphylokinase at 5 μg/mL caused only moderate fibrinogen degradation, indicating that staphylokinase exhibits fibrin specificity. In contrast, SK induced complete fibrinogen breakdown at concentrations over 2.5 μg/mL (Fig 3B). At the concentrations less than 2.5 μg/mL, SK induced less than 30% thrombolysis with extensive fibrinogen breakdown. In the case of t-PA, extensive thrombolysis was observed
without accompanying marked fibrinogen breakdown (Fig 3C).

The thrombolytic efficiencies, which were expressed as the ratio of the degree of thrombolysis versus the degree of fibrinogen breakdown at 6 hours, were compared. With staphylokinase, this ratio was greater than unity at all the concentrations studied in the experiment. The mean value was 2.91 ± 2.17, and the maximum value (6.6) was obtained at a concentration of 2.5 μg/mL. On the other hand, the mean ratio for SK was 0.63 ± 0.33 at all concentrations tested, indicating a lower fibrin-specificity of SK. Thrombolytic efficiencies of t-PA at 6 hours were 6.0 ± 1.6 (the mean value at the t-PA concentration used; 0.56, 1.13, 2.25, 4.5, and 9.0 μg/mL).

The extent of α2-plasmin inhibitor consumption (Fig 2B) was lower in the case of staphylokinase than with SK. Thus, systemic fibrinolytic activation is barely induced by staphylokinase, but specific thrombolysis is induced.

DISCUSSION

Thrombosis is a major life-threatening disease in Western countries, and effective thrombolytic agents may be of clinical value for emergency treatment of such major diseases as acute myocardial infarction, cerebral infarction, or venous thromboembolism. The thrombolytic properties of staphylokinase were investigated in the present study. Staphylokinase responded to FCB-2, and increased the catalytic efficiency at plasminogen activation remarkably. Such characteristics are similar to t-PA, but not observed with SK. Staphylokinase can promote thrombolysis (lysis of fibrin) effectively. That is, the degree of thrombolysis with staphylokinase was much higher than that with SK (Fig 1), and the degree of fibrinogen breakdown with staphylokinase was less prominent than that with SK (Fig 2A). These characteristics of staphylokinase are clearly expressed when the degree of thrombolysis is plotted against the degree of fibrinogenolysis in the same figure (Fig 3). The fibrin-specific thrombolysis by staphylokinase was similar to that of t-PA, without associating fibrinogen breakdown. These activities were independent of the concentration of staphylokinase or t-PA. On the other hand, SK digested fibrinogen mainly with the lesser degree of thrombolysis. A lesser degree of systemic fibrinolytic activation by staphylokinase was also confirmed by the more limited consumption of α2-plasmin inhibitor (Fig 2B) and reduced plasminogen activation.

The thrombolytic efficiency of staphylokinase was also greater than that of SK, indicating that staphylokinase induces fibrin-specific thrombolysis as does t-PA.26 Thus, it is clear that staphylokinase promotes lysis of fibrin more prominently than lysis of fibrinogen. However, the thrombolytic efficiency of staphylokinase was about half of that of t-PA. Further, the concentration of staphylokinase that induced 50% thrombolysis at 3 hours was 2 μg/mL, which is less than 50% of the corresponding concentration of t-PA. However, considering their molecular weights, t-PA was about twice as potent as staphylokinase. Therefore, staphylokinase may induce fibrinogen breakdown if administered to patients. Thus, the further clinical trials with staphylokinase are needed to clarify the fibrin-specific thrombolytic properties of staphylokinase in humans.
t-PA has a molecular weight of 70,000, with 2 Kringle domains whose three-dimensional structure is complex,\textsuperscript{27} and the recovery of t-PA from cell lysate of \textit{E coli} is extremely low. Because staphylokinase is a low molecular weight protein (15,000), production by the recombinant technique with \textit{E coli} is much easier than that of t-PA.\textsuperscript{16} Therefore, staphylokinase with fibrin-specific thrombolytic properties may provide an alternative thrombolytic agent.

REFERENCES

Thrombolytic properties of staphylokinase

O Matsuo, K Okada, H Fukao, Y Tomioka, S Ueshima, M Watanuki and M Sakai