Induction of Sustained Patency After Clot-Selective Coronary Thrombolysis With Hybrid-B, a Genetically Engineered Plasminogen Activator With a Prolonged Biological Half-life

Carla J. Weinheimer, BS; Howard L. James, BS; Narender K. Kalyan, PhD; James Wilhelm, PhD; Shaw Guang Lee, PhD; Paul P. Hung, PhD; Burton E. Sobel, MD; and Steven R. Bergmann; MD, PhD

**Background.** Despite the utility of tissue-type plasminogen activator (t-PA) in eliciting coronary thrombolysis clinically, early reocclusion remains a problem, occurring despite anticoagulation in 5–30% of patients with initially successful recanalization. This study evaluated the utility of Hybrid-B, a molecular variant of t-PA with a prolonged half-life in the circulation, in eliciting coronary thrombolysis and maintaining patency in the presence of a continuing thrombogenic stimulus.

**Methods and Results.** In intact, anesthetized dogs, either 18 mg Hybrid-B over 30 minutes (n=15) or 50 mg t-PA (Activase) over 60 minutes (n=8) was administered starting 60 minutes after left anterior descending coronary artery occlusion was induced with a thrombogenic copper coil. Time to lysis averaged 54±26 (means±SD) minutes and 64±34 minutes with Hybrid-B and t-PA, respectively (p=NS). When Hybrid-B was administered as a bolus (20 mg over 1 minute) to induce a high initial concentration in blood, time to lysis was shortened markedly and averaged 15±5 minutes. Dogs given Hybrid-B by either infusion or bolus exhibited prolonged time to reocclusion (337±192 minutes compared with 192±125 minutes in dogs given t-PA, p<0.03), reflecting maintenance of a subthrombolytic but persistently active concentration of activator in blood. Despite the persistence of Hybrid-B in blood, concentrations of fibrinogen and α2-antiplasmin were not depleted markedly and remained at 77±25 and 56±24%, respectively, of control values.

**Conclusions.** Thus, Hybrid-B, a novel variant of t-PA with unique pharmacokinetic properties, elicits prompt, sustained, and clot-selective coronary thrombolysis. (Circulation 1991;83:1429–1436)

Intravenously administered activators of the fibrinolytic system decrease infarct size, improve left ventricular function, and improve survival of patients with acute transmural myocardial infarction, especially when administered early after the onset of symptoms.1,2 When judged from comparisons of diverse thrombolytic agents, the rapidity and frequency of recanalization seem greater with second- compared with first-generation agents (i.e., recombinant tissue-type plasminogen activator [rt-PA] compared with streptokinase), perhaps because of the clot specificity of second-generation agents and the more modest systemic plasminemia induced.1−4 Nevertheless, failure to induce recanalization is encountered in 15−40% of patients with acute thrombotic occlusion, and early reocclusion occurs in 5−30% of patients in whom recanalization is initially successful.4−8

Reocclusion may reflect incomplete lysis and/or continuing thrombosis stimulated by residual high-grade stenosis and residual clot with elaboration of thrombin. Although antithrombin agents such as heparin and antiplatelet agents such as aspirin extend beneficial effects, they may increase the risk of bleeding and are not universally effective in preventing reocclusion.1−8
We and others have demonstrated that reocclusion can be delayed when tissue-type plasminogen activator (t-PA) is administered in prolonged infusions at subthrombolytic doses. However, this approach requires large amounts of material, vigilant monitoring, and rigorous control of infusion rates. An alternative, theoretically attractive approach entails the use of molecular variants of t-PA designed to exhibit prolonged half-life.

The present study was performed to determine whether such an approach has demonstrable merit. Hybrid-B, a genetically engineered construct of t-PA, was used. It has a single kringle from urokinase (K2) inserted immediately before the double kringle of t-PA (Figure 1). In preliminary studies, we have demonstrated that insertion of the K2 domain prolongs the half-life of the variant compared with that of t-PA.

**Methods**

The experimental protocol was approved by the Washington University Animal Care Committee and conformed to the guiding principles of the American Physiological Society.

**Determination of the Half-Life of Hybrid-B in the Circulation**

Pharmacokinetic studies were performed to define the half-life of antigen and the functional activity of activators after bolus intravenous administration in anesthetized dogs. Nine adult conditioned dogs of either gender were fasted overnight, subcutaneously premedicated with morphine sulfate (1 mg/kg), and intravenously anesthetized with sodium pentothal (12.5 mg/kg) and α-chloralose (72 mg/kg). After endotracheal intubation, ventilation with room air was maintained with a Harvard respirator. Catheters were placed in the abdominal aorta and vena cava by way of a femoral artery and vein.

Five to 6 mg of either Hybrid-B (n=6) or wild-type rt-PA (Activase, Genentech Corp., South San Francisco, Calif.) (n=3) were diluted in 5 ml saline and administered rapidly by bolus intravenous injection. The Hybrid-B used was expressed in mouse C-127 cells transfected with a bovine papilloma virus vector and purified as described previously. It comprised predominantly single-chain material with a functional activity of 350,000 IU/mg, as determined by enzyme-linked immunosorbent assay (ELISA). The functional activity of wild-type t-PA was approximately 600–650,000 IU/mg. After administration of either Hybrid-B or t-PA, arterial blood samples were acquired for assessment of functional activity. Samples were collected in citrated tubes (containing d-phenylalanine-prolylarginine-chloromethyl ketone [PPACK] [Calbiochem, San Diego, Calif.] at a final concentration of 2 μM to inhibit activation of plasminogen in vitro) at 1-minute intervals for 3 minutes and 5, 7, 10, 15, 20, and every 30 minutes thereafter for a total of 180 minutes after injection. After maintenance of samples for 1 hour at 4°C, samples were centrifuged at 800g for 10 minutes at 4°C. Plasma was aspirated and frozen at −70°C until assayed with solid-phase fibrin-tissue immunonassay. In addition, blood samples for determination of the concentration of t-PA or Hybrid-B antigen were collected in Vacutainer tubes containing sodium citrate. Samples were promptly centrifuged at 800g for 10 minutes at 4°C. Plasma was aspirated and stored at −70°C until assayed by ELISA as described below.

**Induction of Thrombolysis**

To compare the efficacy of hybrid-B and t-PA regarding induction of thrombolysis and maintenance of coronary patency, 23 dogs were anesthetized with the same regimen that was used for studies of half-life. After catheterization of the femoral artery and vein, a modified Amplatz (USCI, Billerica, Mass) catheter was introduced into the left common carotid artery. Arterial pressure and the electrocardiogram were monitored and recorded with a Gould (Centerville, Ohio) two-channel recorder. Before induction of coronary occlusion, a loading dose of 1 mg/kg lidocaine was given intravenously, which was followed by a continuous infusion of 50 μg/kg per minute. No antiocoagulants or antiplatelet agents were administered.

A thrombogenic copper coil was used to induce coronary thrombosis, as previously described in detail. After control arteriography, a 0.021-in. guide wire was advanced into the left anterior descending coronary artery and the Amplatz catheter was withdrawn. A copper coil (2 mm i.d., 3 mm o.d., 5 mm in length) was advanced over the guide wire with a small flexible catheter until the coil lodged in the left anterior descending coronary artery past the first diagonal branch. The guide wire was then removed, and the intracoronary catheter was positioned 2–3 cm proximal to the coil and used for sequential injections of contrast medium for documentation of occlusion or patency. All arteriograms were obtained by gently injecting 1–2 ml Omnipaque 350 (Winthrop Pharmaceuticals, New York, N.Y.) contrast dye and were recorded on x-ray film for subsequent analysis. Complete occlusion occurred within 15 minutes after...
insertion of the coil and was documented fluoroscopically. Thrombi were allowed to stabilize for 60 minutes. Coronary arteriography was repeated to confirm persistence of complete occlusion.

Sixty minutes after induction of complete coronary occlusion, 15 dogs were given 18 mg i.v. Hybrid-B. To promptly induce a high blood level, 10% of the total dose was given as a bolus followed by an infusion of 25 µg/kg/min for a total of 30 minutes. For comparison, eight other dogs were given 50 mg of wild-type human rt-PA (10% administered as a bolus followed by an infusion of 31 µg/kg/min for 60 minutes). Although the amount of rt-PA used may seem large, preliminary studies documented the need to use this dose regimen to consistently and thoroughly induce coronary thrombolysis and elicit concentrations in plasma simulating those seen clinically (see “Discussion”). Angiograms were obtained again as soon as the occurrence of ventricular ectopy indicative of reperfusion was noted on the electrocardiogram or when lysis was evident fluoroscopically. After initial lysis, fluoroscopy was performed at 30-minute intervals to define patency. Angiography was repeated to document reocclusion.

Each arteriogram was assessed by three independent readers. The time and dose of Hybrid-B or rt-PA necessary to induce thrombolysis were recorded as was the time to reocclusion. After angiographic documentation of reocclusion, cardiac arrest was induced with an overdose of anesthesia and saturated KCl, and the coronary artery was dissected for macroscopic confirmation of the position and presence of clotting.

During the course of each study, arterial blood samples were sequentially obtained for analysis of Hybrid-B and rt-PA functional activity and antigen. To systemically define the extent of activation of the fibrinolytic system, blood samples were also obtained for assay of fibrinogen and α2-antiplasmin as previously described.
additional dogs were studied. In six, 20 mg i.v. Hybrid-B was administered over a 1-minute interval 60 minutes after coronary occlusion, and in five, 20 mg i.v. t-PA was administered for 1 minute. All other aspects of the protocol were identical to those in the infusion studies.

Biochemical Procedures

Plasminogen activator antigen was assayed by ELISA with commercially available reagents (American Diagnostica, Greenwich, Conn.). The functional activity of plasminogen activators was determined with a chromogenic substrate and the previously described solid-phase fibrin absorption procedure. Fibrinogen was assayed spectrophotometrically by the sodium sulfite precipitation procedure, and α2-antiplasmin was assayed colorimetrically. All results are expressed as percentages of values before in vivo administration of Hybrid-B or t-PA.

Statistical Methods

Values are expressed as mean±SD. Differences were compared with *t* tests for independent samples
Half-life

Hybrid-B exhibited a markedly prolonged half-life in blood compared with that of t-PA. The α-phase of antigen clearance revealed a y intercept of 63% and an average half-time of clearance (t_{1/2}) of 3.4 minutes. The β-phase showed a y intercept of 49% and an average t_{1/2} clearance of 48.7 minutes. By comparison, t-PA exhibited an α-phase with a y intercept of 91% of peak with a t_{1/2} of 2.3 minutes and a β-phase with a y intercept of 9% of peak with a t_{1/2} of 9.8 minutes (Figure 2, p<0.01 for each comparison).

The functional activity of Hybrid-B compared with that of t-PA was prolonged as well, with an α-phase of 55% of peak and a t_{1/2} of 2.2 minutes and a β-phase of 43% and a t_{1/2} of 24.8 minutes. In contrast, after administration of t-PA by bolus injection, functional activity declined, with an α-phase of 63% of peak and a t_{1/2} of 1.4 minutes and a β-phase of 37% and a t_{1/2} of 3.9 minutes (Figure 2, p<0.01 for each comparison).

The area under the antigen time–concentration curve, obtained by integrating the area under the curve to the last time point (180 minutes), was 7.8-fold greater for Hybrid-B than for t-PA. For functional activity, the integral of the area under the curve was 5.2-fold greater for Hybrid-B than for t-PA (p<0.01 for each comparison).

Thrombolysis

The first eight dogs given Hybrid-B were studied only to evaluate the efficacy of the agent in inducing lysis. In the seven dogs subsequently given Hybrid-B, the time to reocclusion was determined as well. Figure 3 depicts arteriograms from one representative animal.

The time to lysis (measured from the onset of drug administration) and the time to reocclusion (measured from the end of infusion) were evaluated with the use of serial arteriograms. Time to lysis was similar, with infusions of Hybrid-B (n=15) and t-PA (n=8) averaging 54±26 minutes and 64±34 minutes, respectively (Figure 4). In six dogs, time to lysis was evaluated after bolus administration of 20 mg i.v. Hybrid-B for 1 minute. Lysis occurred more rapidly when activators were given as a bolus for 1 minute than when administered by infusion. The average time to lysis was 15±5 minutes after bolus administration of Hybrid-B and 34±8 minutes after bolus administration of 20 mg t-PA, excluding data from one of five dogs that received t-PA but in which thrombolysis was not induced (p<0.002 and p<0.05 compared with time to lysis with Hybrid-B or t-PA given by infusion).

Dogs given Hybrid-B by either bolus injection for 1 minute or infusion for 30 minutes (n=10) exhibited prolonged time to reocclusion compared with values in dogs given t-PA (n=8). The average time to reocclusion in dogs given Hybrid-B was 337±192 minutes compared with 192±125 minutes in dogs given t-PA (p<0.03) (Figure 4). Time to reocclusion averaged 380±69 minutes in dogs given Hybrid-B by bolus injection for 1 minute (n=3), a value similar to that of Hybrid-B infusion (319±123 minutes, n=7) and prolonged compared with time to reocclusion in dogs receiving 20 mg t-PA for 1 minute (134±81 minutes, p<0.08).

Activation of the Fibrinolytic System

Because of the prolonged half-life of Hybrid-B in the circulation, infusion resulted in peak antigen levels of 5,129±2,355 ng/ml that were approximately twice as high as those found after infusion of t-PA (3,086±2,006 ng/ml) (p<0.05, Figure 5), even though dogs were given almost threefold as much t-PA as Hybrid-B. Peak functional activity with Hybrid-B averaged 2,144±757 compared with 1,435±1,440 IU/ml with t-PA (Figure 5).
Functional activity as a function of time after onset of infusion is shown in Figure 6. The persistence of functional activity with Hybrid-B compared with t-PA is apparent. Prolongation of the time to reocclusion correlated closely with persistence of functional activity ($r = -0.90, p < 0.006$).

To determine whether the prolonged activity in the blood of Hybrid-B was associated with an increase in systemic fibrinolysis, sequentially obtained blood samples were assayed for fibrinogen and $\alpha_2$-antiplasmin. Despite the relatively high and sustained amounts of Hybrid-B activity, systemic lytic effects were modest (Figure 7). The nadir values of fibrinogen and $\alpha_2$-antiplasmin were $77\pm25\%$ and $56\pm24\%$ of preinfusion values in dogs given Hybrid-B by infusion for 30 minutes, values slightly higher than those observed after infusion of t-PA ($66\pm27$ and $37\pm23\%$). Concentrations of fibrinogen and of $\alpha_2$-antiplasmin were comparably maintained after bolus administration of Hybrid-B for 1 minute ($76\pm11$ and $43\pm12\%$ of predrug levels, respectively).

**Discussion**

The results of this study indicate that Hybrid-B, a genetically engineered variant of t-PA that is designed to exhibit a prolonged half-life in the circulation, induces prompt, clot-selective coronary thrombolysis and sustained patency. Factors potentially contributing to the prolonged half-life of Hybrid-B compared with that of t-PA include modifications of expression of high mannose residues and conformational differences affecting recognition by hepatic receptors (Figure 1). Because of the prolonged half-life, reocclusion was delayed with Hybrid-B despite the presence of a continuing stimulus to thrombosis. Thus, reocclusion occurred only at a time when functional activity had substantially declined, which was consistent with results of previous studies with t-PA from our laboratory. However, because binding of plasminogen activators to clot-associated fibrin is persistent, functional activity in plasma may underestimate the persistence of activity in the clots themselves.

Even with the high plasma levels achieved and the protracted functional activity of Hybrid-B, depletion of fibrinogen was only modest.

**Technical Considerations**

The doses of t-PA used in the present study were selected to induce thrombolysis promptly and as completely as possible while eliciting concentrations in plasma simulating those seen clinically, based on results in 12 preliminary studies in which diverse doses of commercially available t-PA were evaluated. Although previous studies from our laboratory have demonstrated lysis with lower doses of t-PA than those used in the present study, when dogs with inserted coils were given 10 mg or less, low peak antigen concentrations or the lack of complete thrombolysis occurred ($n=9$). With doses of 18 mg (equivalent to the dose of Hybrid-B used), only partial lysis was seen ($n=3$). With doses of 50 mg given for 1 hour (with 10% as a bolus), thrombolysis was apparently complete, and plasma concentrations of antigen were similar to those seen clinically with doses greater than or equal to 60 mg. Moreover, peak levels of functional activity were roughly equivalent (Figure 5). The integral of the functional activity of 18 mg Hybrid-B administered by infusion was 1.2-fold that of 50 mg t-PA administered by infusion, as would be anticipated based on their pharmacokinetic proper-
ties, which further supported the dose regimen used. We attribute the need to infuse relatively large amounts of t-PA to modifications that have been made to the present clinically formulated agent since the time of performance of our initial studies.  

When identical doses (by mass) of activator were given as a bolus for 1 minute, time to lysis was shortened and time to reocclusion prolonged with Hybrid-B, further supporting the concept that activators with prolonged plasma clearance are advantageous. Our determinations of time to lysis and time to reocclusion were made in experimental animals in which a thrombogenic copper coil remained in place in the left anterior descending coronary artery. Although results with such preparations have presaged those from patients in studies of fibrinolytic agents with and without adjunctive agents, they do not mimic atherosclerotic coronary artery disease in patients. Neverthe-

less, residual stenosis is common after successful thrombolysis in patients and may serve as a somewhat analogous nidus for recurrent thrombosis.

Clinical Implications

The results of this study indicate that the use of Hybrid-B, a novel variant of t-PA constructed to exhibit prolonged clearance from the blood and concomitantly prolonged biological activity, permits prompt induction of coronary thrombolysis with doses markedly lower than those required with t-PA. Furthermore, the genetically engineered variant prolongs the time to reocclusion. Use of more modest doses of plasminogen activators is attractive because it may permit reduction of costs in the clinical setting.

Hybrid-B may permit rapid induction of thrombolysis with sustained patency without the need for prolonged infusions. Its pharmacokinetic profile suggests that a bolus injection may be sufficient to induce lysis promptly in patients because of the high initial concentrations anticipated and the slow clearance. Therefore, treatment may be simplified. Persistence of relatively high concentrations would be anticipated to prevent early reocclusion as well. Despite its prolonged clearance from the circulation, it does not induce systemic lytic effects with therapeutically effective doses. Accordingly, it is a particularly attractive agent for evaluation in patients.

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References

influence of maintained infusion on reocclusion rate. *Am J Cardiol* 1987;60:231–237


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