In Vivo Anticoagulant Properties of a Novel Nucleotide-Based Thrombin Inhibitor and Demonstration of Regional Anticoagulation in Extracorporeal Circuits

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Using a novel in vitro selection/amplification technique, we have recently identified a new class of thrombin inhibitors based on single-stranded DNA oligonucleotides. One oligonucleotide, GGTTGGTGTGGTTGG (thrombin aptamer), showed potent anticoagulant activity in vitro. We have initiated pharmacologic studies in cynomolgus monkeys to study the thrombin aptamer's in vivo anticoagulant properties. Upon infusion of the thrombin aptamer, anticoagulation was rapidly achieved, with a plateau reached within 10 minutes. There was a linear dose-response relationship between thrombin aptamer infusion rate and prolongation of plasma prothrombin time. Ten minutes after the infusion was stopped, no prolongation of prothrombin time was observed, indicating that the thrombin aptamer has an extremely short in vivo half-life, estimated to be 108 ± 14 seconds. In addition, inhibition of thrombin-induced platelet aggregation in platelet-rich plasma was observed ex vivo without an effect on collagen-induced aggregation, indicating that the inhibition was specific for thrombin and not due to a nonspecific inhibitory effect on platelets. To exploit the short in vivo half-life of the thrombin aptamer, its ability to achieve regional anticoagulation in an extracorporeal hemofiltration circuit in sheep was tested. Doubling of the prothrombin time in the circuit was observed, whereas the systemic prothrombin time was minimally prolonged. We conclude that the thrombin aptamer is a potent anticoagulant in vivo, and specifically inhibits thrombin-induced platelet aggregation ex vivo. The rapid onset of action and short half-life in vivo suggest that the thrombin aptamer may be useful in anticoagulation with extracorporeal circuits and may have distinct advantages in certain acute clinical settings.

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THROMBIN PLAYS a pivotal role in hemostasis. It activates the cofactors factor V and VIII in the clotting cascade and catalyzes the conversion of soluble fibrinogen to insoluble polymeric fibrin. It is also a potent activator of platelets. Heparin accelerates the inhibition of thrombin by its natural plasma inhibitor antithrombin III (ATIII) and is widely used in the treatment of thrombotic disorders. However, in addition to bleeding complications, heparin has significant clinical limitations and undesirable side effects. Because heparin is dependent on ATIII for its anticoagulant function, the effectiveness of heparin is limited in clinical situations associated with significant consumption and reduction in plasma ATIII level, such as disseminated intravascular coagulation. Heparin-associated thrombocytopenia, an immune mediated disorder occurring in approximately 5% of patients exposed to heparin, is also sometimes complicated by life-threatening arterial thrombosis. Another limitation of heparin is its relative ineffectiveness in the treatment of arterial thrombosis. This heparin resistance may be related to the release of platelet-factor 4 (PF4) by activated platelets, which neutralizes heparin's anticoagulant function, and the relative inaccessibility of cell surface or clot-bound thrombin to the heparin-ATIII complex. Thus, a strong impetus exists for the development of direct thrombin inhibitors that can circumvent some of the limitations of heparin anticoagulation.

We have recently identified a new class of thrombin inhibitors based on specific single-stranded (ss) DNA oligonucleotides, using a novel selection and amplification method. In general, the technique involves the application of a large population of randomly generated ssDNA sequences to a specific target. A subpopulation of the ssDNA molecules, selected for specific target binding properties, is amplified by polymerase chain reaction (PCR) and the selection process is repeated. This iterative selection and amplification process allows the rapid screening of an extremely large number (>10^15) of distinct molecules for the subset of oligonucleotides, termed aptamers, that binds a specific target. Significantly, the targets need not normally interact with nucleic acids, and using this technique, we identified ssDNA aptamers that bind specifically to thrombin but not other serine proteases. The selected aptamers inhibited thrombin-catalyzed fibrin clot formation in vitro using either purified fibrinogen or human plasma. Sequence analysis of the aptamer clones showed a highly conserved 14- to 17-base region. A synthetic oligonucleotide, GGTTGGTGTGGTTGG (thrombin aptamer), incorporating the consensus sequence, displayed potent thrombin inhibitory activities in vitro.

Because the ssDNA thrombin aptamer represents a novel class of thrombin inhibitor, it is important to test its anticoagulant properties in vivo and to study its pharmacokinetic parameters. In this study, we show that the thrombin aptamer is a potent anticoagulant in a primate model. The initial pharmacokinetic data show that the thrombin aptamer has a rapid onset of action and short half-life in vivo, suggesting that the thrombin aptamer may have distinct advantages over other thrombin inhibitors in certain acute clinical settings.

MATERIALS AND METHODS

Preparation of thrombin aptamer. The ssDNA molecule GGTTGGTGTGGTTGG (thrombin aptamer) was prepared by solid-phase phosphoramidite chemistry on a Biosearch 8800 synthe-
jugular venous catheters. An Amicon mini-flow hemofiltration catheter was inserted for blood sampling. Access for arterial-venous general anesthesia throughout the experiment. A femoral venous line was connected to the carotid arterial and jugular venous catheters. Constant blood flow was maintained at 50 mL/min by an in-line centrifugal pump. The thrombin aptamer was infused into the arterial limb of the hemofiltration circuit at a constant rate calculated to achieve 3 μmol/L final concentration in the circuit. Blood samples for prothrombin time (PT) analysis were drawn from the circuit just before the hemofilter. For comparison, timed samples were drawn from the femoral venous catheter to measure the extent of systemic anticoagulation. Hemofiltration under these conditions was performed for 2 hours. Similarly, hemodialysis was performed at a blood flow rate of 100 mL/min using a CA-90 hollow fiber hemodialysis cartridge (Baxter, McGaw Park, IL).

**PT assays.** Blood samples were centrifuged (2,000g for 5 minutes) and the plasma separated for standard PT assays. PT times were measured using an automated fibrometer (Becton Dickinson, Mountain View, CA) using Sigma (St Louis, MO) reagents as per the manufacturer’s specifications.

**Platelet aggregometry.** Platelet aggregation was performed with platelet-rich plasma (PRP) (150g for 20 minutes) in the standard manner (incubated at 37°C for 5 minutes before assay) using a Chronolog dual-channel aggregometer (Chrono-Log Corp, Havertown, PA). Human thrombin (1 U/mL) (Haematologics, Essex Jet, VT) and collagen (Chrono-Log) were used as platelet agonists.

**RESULTS**

**In vitro dose response of thrombin aptamer in plasma.** The relationship between thrombin aptamer concentration and prolongation of PT in cynomolgus monkey plasma was determined in vitro (Fig 1). The results show a linear dose response in the range studied with a doubling of the control PT at approximately 2 μmol/L thrombin aptamer in plasma. This standard curve was used to determine the levels of thrombin aptamer in plasma at time points during and after IV infusion of thrombin aptamer.

**Constant rate infusion of thrombin aptamer in vivo.** The time course of thrombin aptamer-mediated anticoagulation during a constant rate IV infusion was studied (Fig 2). Thrombin aptamer was infused into four animals at a rate of 0.3 mg/kg/min over a period of 1 hour. Saline alone was infused into one animal as a control. Anticoagulation was rapidly achieved, with a plateau reached within 10 minutes at 1.7 to 2.0 times control PT, corresponding to calculated in vivo plasma concentrations of 1.5 to 2.1 μmol/L. When the infusion was stopped, there was a rapid reversal of the anticoagulant effect such that at 10 minutes after the infusion, the PT had returned to baseline. The data indicated that the in vivo half-life of the thrombin aptamer is extremely short. Based on an exponential fit of the data, from the termination of infusion to 300 seconds, the T½ was determined to be 108 ± 14 seconds.

**Variable rate infusion of thrombin aptamer in vivo.** To determine if the anticoagulant effect was dose dependent, an increasing, variable dose infusion was studied. Thrombin aptamer was infused into two animals at an initial rate of 0.075 mg/kg/min. The infusion rate was doubled every 15 minutes over a period of 1 hour. There was a rapid response in PT to the increasing dose in thrombin aptamer (Fig 3A) and a linear dose-response relationship between thrombin aptamer infusion rate and prolongation in PT in plasma was observed (1.2 to 2.2 times control PT) (Fig 3B).

**Thrombin aptamer inhibition of specific thrombin-in-
IN VIVO ANTICOAGULATION BY THROMBIN APTAMER

Fig 1. Relationship between thrombin aptamer concentrations and prolongation in plasma PT in vitro from two cynomolgus monkeys. Control PT was 12 to 13 seconds.

duced platelet aggregation ex vivo. The effect of thrombin aptamer on thrombin- or collagen-induced platelet aggregation in PRP was determined ex vivo in samples collected from the variable dose infusion study. Even at the lowest dose (0.075 mg/kg/min), thrombin aptamer effectively inhibited thrombin-induced platelet aggregation (Fig 4B). It should be noted that at this low dose of thrombin aptamer, platelet shape change was observed as evidenced by a decrease in light transmission after thrombin addition, indicating that there was sufficient thrombin to bind to the platelet thrombin receptor to cause partial platelet activation. This is consistent with the reduced amplitude in the aggregation tracing upon subsequent collagen stimulation, suggesting that platelets were partially activated after thrombin stimulation and thus became partially refractory to collagen (Fig 4B). At higher rates of thrombin aptamer infusion, shape change was completely abolished and the full platelet aggregation response to collagen was observed (Fig 4D and E). Even at the highest dose of thrombin aptamer, platelets responded normally to collagen, indicating that the thrombin aptamer inhibitory effect is specific for thrombin interaction with the platelet thrombin receptor, and not due to nonspecific inhibitory effects on platelets. Similar to the anticoagulant effect, platelets responded normally to throm-

Fig 2. Time course of thrombin aptamer-mediated anticoagulation (PT) during IV infusion. Thrombin aptamer was infused into four animals at a rate of 0.3 mg/kg/min over a period of 1 hour (○) (mean ± SD, n = 4). Saline alone was infused into one animal as a control (○). Blood samples were collected from each animal at different time points and assayed. Anticoagulation was rapidly achieved with a plateau reached within 10 minutes at 1.7 to 2.0 times control PT. Ten minutes after the infusion was stopped, no prolongation of PT was observed, indicating a very short in vivo half-life of the thrombin aptamer.

Fig 3. (A) Dose-response of increasing thrombin aptamer infusion and prolongation in plasma PT. Thrombin aptamer was infused into two animals at a rate of (A) 0.075 mg/kg/min, (B) 0.15 mg/kg/min, (C) 0.30 mg/kg/min, and (D) 0.60 mg/kg/min. Blood samples were collected at different time points from each animal (○ and □) and assayed. Control PT was 12 to 13 seconds. The PT responded linearly to increasing dose (1.2 to 2.2 times control PT).
Fig 4. (A through F) Platelet aggregation studies showing the effect of IV infusion of thrombin aptamer at different rates on thrombin-induced (T) or collagen-induced (C) platelet aggregation in PRP ex vivo.

Hematology and serum chemistry. A decrease in the hematocrit (15% to 17% decrease from 100% baseline) was observed in all three groups of animals (constant rate infusion, variable rate infusion, and saline control) that is most likely related to hemodilution and multiple blood drawing. A significant increase in the white blood cell (WBC) count was also seen in all three groups at 30 minutes postinfusion (mean WBC 23,820/μL for animals receiving thrombin aptamers v 21,100/μL for the saline control animal), probably due to stress-related neutrophilia. Neutrophil and WBC counts returned to near normal levels 24 hours after infusion. There was a moderate increase (2 to 3 times baseline) in serum creatine phosphokinase (CPK) in all groups, including control, at 30 minutes and 24 hours postinfusion. One animal in the constant thrombin aptamer infusion group had a marked increase in CPK. Electrophoresis of the serum sample showed 99.6% CPK MM isotype, indicating that skeletal muscle was the primary source of this enzyme. Trauma associated with chair restraint and other study procedures could account for the increase in serum CPK. All other serum chemistry values, including electrolytes, liver enzymes, and kidney function tests, were normal after infusion of thrombin aptamer. All the animals tolerated the aptamer infusion without any problems and remained well after the experiments.

Demonstration of regional anticoagulation in sheep hemofiltration. To exploit the short in vivo half-life of the thrombin aptamer, its ability to achieve regional anticoagulation in an extracorporeal circuit was tested. Regional anticoagulation is defined as adequate prolongation of PT within the extracorporeal circuit while maintaining the PT in the systemic circulation at or near baseline. Hemofiltration was performed in the sheep, with the thrombin aptamer infused just proximal to the hemofiltration unit. Clotting times were determined from samples taken simulta-
necessarily from the extracorporeal circuit and from a peripheral vein. There was significant prolongation of the PT in the hemofiltration circuit (PT range, 40 to 45 seconds; PT baseline, 21.7 seconds), whereas the systemic PT remained essentially unchanged or only slightly prolonged (Fig 5), clearly showing regional anticoagulation in an extracorporeal circuit by the thrombin aptamer. Similar results were observed in hemodialysis performed on a separate sheep (data not shown).

DISCUSSION

Using a novel selection and amplification technique, we have recently identified new thrombin inhibitors based on ssDNA oligonucleotides. Because these specific ssDNA oligonucleotides represent a new class of thrombin inhibitor, it is critical to assess their in vivo anticoagulant efficacy and pharmacology. In this study, we have chosen a synthetic 15-mer oligonucleotide (thrombin aptamer), incorporating the active consensus sequence, and infused it intravenously into cynomolgus monkeys. The thrombin aptamer effectively prolonged the PT in a dose-dependent manner and specifically inhibited thrombin-induced platelet aggregation ex vivo (Figs 2 through 4). The animals tolerated the infusion of thrombin aptamer well without clinically evident side effects and the data indicate that it has potent anticoagulant efficacy in vivo.

The thrombin aptamer has a short half-life in vivo of approximately 108 seconds. Previous studies have shown that unmodified ssDNA oligonucleotides have a half-life of less than 1 minute after IV bolus injection in mice or rats. Our data are consistent with those observations. It is unlikely that this short half-life is due to rapid degradation of the ssDNA aptamer by plasma exonuclease activity. In contrast to RNA, ssDNA is much more resistant to exonuclease degradation, and the in vitro half-life of the thrombin aptamer in human plasma has been estimated to be approximately 30 minutes (data not shown). The observation that the thrombin aptamer has a significantly longer plasma half-life in vitro also rules out the possibility that the thrombin aptamer is rapidly inactivated by nonspecific binding to plasma proteins. In addition, the in vivo half-life of the aptamer was not substantially prolonged after a 60-minute infusion as compared with an IV bolus injection (data not shown), suggesting that saturation of nonspecific binding sites in plasma or other compartments in vivo does not play a major role in determining the short half-life. More recently, we have developed an HPLC assay to directly measure the plasma concentration of the thrombin aptamer and have shown that the short half-life directly correlated with decreasing plasma concentration (W. Lee, J. Fishback, J. P. Shaw, personal communication, June 1992).

Previous studies suggest that cellular uptake of unmodified phosphodiester-linked oligonucleotides involves endocytosis, apparently mediated by specific saturable cell surface receptors. Rapid uptake of ssDNA by the liver has been observed after IV bolus injection in mice. Wide tissue distribution of modified oligonucleotides after IV injection has been reported. Whether the rapid clearance of the unmodified ssDNA thrombin aptamer is due to widespread cellular uptake and tissue distribution remains to be determined.

Hemodialysis in the presence of systemic anticoagulation with heparin carries a significant risk of clinically important hemorrhage, with a reported incidence of 10% to 19%. We have exploited the short in vivo half-life of the thrombin aptamer in achieving regional anticoagulation in an extracorporeal hemofiltration circuit, with the PT in the circuit maintained at 2X baseline whereas the systemic PT was minimally prolonged (Fig 5). Regional anticoagulation is desirable in clinical situations in which systemic anticoagulation poses a substantial risk for major hemorrhage, such as patients with uremic pericarditis, acute renal failure, active gastrointestinal bleeding, and recent cardiovascular surgery.

An additional advantage of a potent thrombin inhibitor with a short half-life is obviating the need for reversal of the anticoagulant effect. The anticoagulant effect of heparin is most commonly reversed with protamine sulfate, which is routinely performed after cardiopulmonary bypass surgery. However, protamine sulfate can cause hypotension by direct vasoconstriction, and in some cases severe pulmonary vasoconstriction. An anaphylactoid type reaction, associated with profound shock that is often fatal, has also been described. Presensitization of the susceptible patients to protamine by exposure to protamine in NPH or protamine-zinc insulin has been implicated. A direct thrombin inhibitor with a short half-life that can provide adequate anticoagulation during cardiopulmonary bypass surgery without the subsequent need for reversal will be of considerable clinical interest.

In addition to its potent anticoagulant property, we have recently shown that the synthetic thrombin aptamer inhibits clot-bound thrombin and reduces arterial thrombus formation in an ex vivo whole artery angioplasty model. In conclusion, rapid in vivo clearance of the thrombin aptamer provides an opportunity for drug development in certain clinical settings in which regional anticoagulation in
extracorporeal circuits is desirable (eg, hemodialysis and hemofiltration). This aptamer, composed of only natural nucleotides, is nontoxic even when administered at doses that achieve systemic anticoagulation. Finally, this aptamer provides a lead for further drug design by a variety of chemical modifications, including the phosphodiester backbone, the base, and the sugar moieties, to enhance its pharmacologic and therapeutic properties. These modifications are guided by a knowledge of the tertiary structure of the thrombin aptamer. Thus, aptamer technology represents a powerful new approach for rapid drug discovery and development.

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