A Novel Nucleotide-Based Thrombin Inhibitor Inhibits Clot-Bound Thrombin and Reduces Arterial Platelet Thrombus Formation

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A novel thrombin inhibitor based on single-stranded (ss) deoxynucleotides with the sequence GGTGGTGTGGTGGTGGT (thrombin aptamer) has been recently discovered. In this study, we tested its efficacy in inhibiting clot-bound thrombin activity and platelet thrombus formation in an ex vivo whole artery angioplasty model. The thrombin aptamer showed a specific dose-dependent inhibition of thrombin-induced platelet aggregation (0.5 U/mL) in human platelet-rich plasma, with an IC50 of approximately 70 to 80 nmol/L. In an in vitro clot-bound thrombin assay system, heparin, used at clinically relevant concentrations of 0.2 U/mL and 0.4 U/mL, was ineffective in inhibiting clot-bound thrombin (6.5% and 34.9% inhibition at 0.2 U/mL and 0.4 U/mL, respectively). In contrast, the thrombin aptamer at an equivalent anticoagulant concentration inhibited clot-bound thrombin (79.7% inhibition). In an ex vivo whole artery angioplasty model, the thrombin aptamer markedly suppressed the generation of fibrinopeptide A (FPA), whereas heparin at 2 U/mL was ineffective. Compared with a scrambled ssDNA control, the thrombin aptamer reduced platelet deposition by 34.5% and 61.3% (5% and 11%) (mean ± SEM, n = 4, 0.05) at low shear rates (~200 s⁻¹) and high shear rates (~850 s⁻¹). Thrombin aptamers based on ssDNA molecules represent a new class of thrombin inhibitors with potent anticoagulant and antithrombotic properties.

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MATERIALS AND METHODS

Preparation of thrombin aptamer. The ssDNA molecule GGTGGTGTGGTGGTGGT (thrombin aptamer) and scrambled ssDNA control GGTGGTGTGGTGGTGGT were prepared by solid-phase phosphoramidite chemistry on a MilliGen/Biosearch 8700 synthesizer (Burlington, MA) using commercially available phosphoramidite monomers (Glen Research, Sterling, VA) as previously described. The synthetic DNA was purified by polyacrylamide reverse phase high performance liquid chromatography (HPLC), detritylated, and secondarily purified using C18 reverse phase HPLC to greater than 97% homogeneity by ion exchange analysis. A 20 mg/mL stock solution was prepared by dissolving the thrombin aptamer in 20 mmol/L sodium phosphate buffer (pH 7.4). The concentration of the aptamer was determined by UV absorbance at 260 nm (6.98 nmol/OD).

Radioimmunoassay of fibrinopeptide A (FPA). FPA in plasma was measured using a radioimmunoassay (RIA) kit purchased from American Diagnostica Inc (Greenwich, CT) following the manu-
A: Thrombin Aptamer  
T: a-Thrombin (0.5 U/mL)  
S: Scrambled Aptamer (1.45 μM)  
C: Collagen (2.4 μg/mL)

Fig 1. Effect of thrombin aptamer on platelet aggregation in human platelet-rich plasma in vitro. Thrombin aptamer (0.07 to 1.45 μmol/L) showed dose-dependent inhibition of thrombin-induced (0.5 U/mL) platelet aggregation (B-F). Even at the highest dose (1.45 μmol/L) tested, thrombin aptamer had no effect on collagen-induced aggregation (F). Scrambled aptamer control (1.5 μmol/L) had no inhibitory effect on thrombin-induced aggregation (G). The results represent one of four similar experiments.

Manufacture's directions. A separate standard curve was generated for each experiment.

Generation of FPA by clot-bound thrombin. Fibrin clots were formed by mixing fresh citrated human plasma (75 μL) with equal volumes of 25 mmol/L CaCl2 in the wells of 48-well tissue culture plates and incubated at 37°C for 60 minutes. The fibrin clots were then washed with 1-mL aliquots of 50 mmol/L Tris-HCl, 0.1 mmol/L NaCl, pH 7.4 (TBS) for eight times over a period of 16 hours to remove any FPA trapped in the clots. Fresh citrated plasma (200 μL) or purified fibrinogen (2 mg/mL) in TBS was added to the washed fibrin clot in the wells and incubated at 37°C. At timed intervals, 100-μL aliquots of plasma or fibrinogen solution were removed and assayed for FPA. The 100-μL aliquots were mixed immediately with 300 μL ice-cold 100% ethanol to precipitate the unreacted fibrinogen. The mixtures were centrifuged at 15,000×g for 5 minutes and the ethanol supernatants evaporated to dryness in a Savant Speed-Vac concentrator (Farmingdale, NY), reconstituted to the original volumes with distilled water, and assayed for FPA by RIA. To determine the amount of FPA derived from trapped FPA in the washed clots, TBS (200 μL) was placed over the clots and 100 μL removed at different time points and assayed for FPA. To exclude the possibility that the detected thrombin activity was caused by thrombin diffusing from the clot surface rather than clot-bound thrombin, aliquots of TBS incubated over the fibrin clot were removed after various periods of time and tested for thrombin activity using a chromogenic substrate. The antithrombin effect of the thrombin aptamer was compared with sodium heparin (1,000 U/mL, Elkins-Sinn, Inc, Cherry Hill, NJ). Duplicate or triplicate samples were obtained for each time point in each experiment.

Ex vivo whole artery angioplasty model. A recently described ex vivo whole-artery angioplasty model was used.12 Briefly, freshly dissected rabbit aortas were mounted in a perfusion chamber and one half of the arterial segment was dilated with a standard balloon catheter (three 30-second balloon inflations at 6 atm) with the uninjured half serving as a control. The vessels were perfused with heparinized (2 U/mL) human whole blood, in the presence or absence of thrombin aptamer, at physiologic pressure and shear rates of ~200 s⁻¹ (low shear) or ~850 s⁻¹ (high shear) for 30 minutes. Previous studies have shown that maximal platelet deposition was reached at 30 minutes.12 Shear rates were determined using the formula \(4 \times \text{Flow}/\pi \text{(radius)}\).13 The vessel diameters were measured using a two-dimensional ultrasound device. Aliquots were removed at different time points for FPA determinations. Platelet deposition on the injured and uninjured portions of the vessels was determined using 111In-labeled platelets as previously described.13 In these studies, mean platelet deposition on the injured vascular segment was 9.2 × 10⁶ platelets/cm² and 20.2 × 10⁶ platelets/cm² at low and high shear, respectively, and 1.0 × 10⁶ platelets/cm² on the uninjured portion.

Platelet aggregation studies. Platelet aggregation studies were performed with citrated human platelet-rich plasma (PRP) using a Chronolog two-channel aggregometer (Chrono-Log Corp, Haverc-
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The thrombin aptamer showed a dose-dependent inhibition of thrombin-induced platelet aggregation (0.5 U/mL) in human PRP (Fig 1B–F). Significant partial inhibition was observed at 70 to 150 nmol/L of thrombin aptamer (Fig 1B,C). At 0.72 μmol/L, there was complete inhibition of aggregation while platelet shape change was evident (Fig 1E) suggesting that there was sufficient residual active thrombin to bind to the platelet thrombin receptors and cause partial platelet activation. At a higher concentration of thrombin aptamer, shape change was abolished, whereas platelet response to collagen stimulation remained normal (Fig 1F) indicating that the aptamer inhibitory effect was specific for thrombin and not due to nonspecific inhibition of platelets. Scrambled aptamer control did not show any inhibitory effect on thrombin-induced platelet aggregation (Fig 1G). The concentration of thrombin aptamer required to achieve 50% inhibition (IC₅₀) of platelet aggregation, as determined by planimetry of the aggregation tracings, was estimated to be approximately 70 to 80 nmol/L (Fig 2).

Thrombin aptamer inhibited clot-bound thrombin activity in vitro. To determine if the thrombin aptamer effectively inhibited clot-bound thrombin, an in vitro clot-bound thrombin assay system was used. Fibrin clots were formed by recalcification of citrated plasma and washed extensively to remove any FPA trapped within the clots. Plasma was then placed over the washed clots and the generation of FPA determined. There was a time-dependent generation of FPA in plasma that was not caused by release of FPA trapped within the washed clots, because the addition of buffer over the clots produced only a minimal amount of FPA (6.48 ± 1.55 nmol/L at 60 minutes, mean ± SEM, n = 8) (Fig 3).

The endogenous FPA level was 4.22 ± 1.85 nmol/L (mean ± SEM, n = 8) in these studies. The measured thrombin activity was caused by clot-bound thrombin and not thrombin diffusing from the clot surface, because buffer incubated over the clot did not show any detectable enzymatic activity as measured by a chromogenic substrate (data not shown). The data show that fibrin clots retain a significant amount of active thrombin capable of interacting with plasma fibrinogen to generate FPA, which is in agreement with recent published results.

The efficacy of the thrombin aptamer in inhibiting clot-bound thrombin was compared with heparin and the synthetic thrombin inhibitor D-phenylalanyl-L-prolyl-L-arginyl chloromethyl ketone (PPACK). Heparin was used at clinically relevant concentrations of 0.2 U/mL and 0.4 U/mL, which prolonged the activated partial thromboplastin time (aPTT) to approximately 2.0 and 3.4 times baseline, respectively (Fig 4). At this concentration, heparin completely inhibited the activity of free thrombin (0.05 nmol/L) added to plasma, which generated similar amounts of FPA as in the clot-bound thrombin system (data not shown). However, heparin at 0.2 U/mL was ineffective in inhibiting clot-bound thrombin activity (6.5% inhibition),
and at 0.4 U/mL, it had only a modest inhibitory effect (34.9% inhibition) (Fig 5). In contrast, the thrombin aptamer significantly inhibited clot-bound thrombin (79.7% inhibition), as did PPACK (95% inhibition). The thrombin aptamer and PPACK were used at a concentration (3 μmol/L) that had approximately equivalent anticoagulant effect in vitro when compared with heparin prolonging the aPTT to 2.8 times baseline for both PPACK (data not shown) and thrombin aptamer (Fig 4). The data indicate that clot-bound thrombin is protected from inhibition by heparin-antithrombin III (ATIII) at clinically therapeutic heparin concentrations, whereas it is susceptible to inhibition by the thrombin aptamer.

To show that the measured thrombin activity was not caused by clot-bound factor FXa that generated soluble thrombin from plasma prethrombin, fibrinogen solution instead of plasma was placed over the washed clots and the generation of FPA determined. A substantial amount of FPA was generated in the supernate (210 ± 4.2 nmol at 60 minutes of incubation, mean ± SEM, n = 5), which was inhibited by the thrombin aptamer in a dose-dependent manner (Fig 6).

In these studies, the thrombin aptamer was used at a concentration (3 μmol/L) that prolonged the aPTT to approximately 2.8 times baseline. Previous studies in animal models have shown that these concentrations of thrombin aptamer can be safely achieved by continuous intravenous infusion.

Thrombin aptamer suppressed FPA generation and reduced platelet deposition in an ex vivo whole-artery angioplasty model. To extend these in vitro observations to a more clinically relevant system, the effect of the thrombin aptamer on FPA generation in an ex vivo whole-artery angioplasty model was studied. In this model, heparinized human whole blood was perfused over a balloon-angioplasty-injured rabbit aorta. Even in the presence of high-dose heparin (2 U/mL), a significant amount of FPA was generated (Fig 7), suggesting that fibrin-exposed or exposed matrix-bound thrombin was resistant to heparin-ATIII, which is consistent with previous published studies. Similar to the in vitro data, the thrombin aptamer markedly suppressed the generation of FPA (Fig 7).
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We have also tested the efficacy of the thrombin aptamer in preventing platelet thrombus formation over the injured arterial segments (Fig 8). Compared with the scrambled ssDNA control, the thrombin aptamer reduced platelet deposition by 34.5% ± 5% (mean ± SEM, n = 4, P = .09) at low shear rates (~200 s⁻¹). At higher shear rates (~850 s⁻¹), which more closely approximate those encountered in stenosed coronary arteries, the inhibition was more apparent (61.3% ± 11%, mean ± SEM, n = 4, P = .05). The extent of inhibition by the thrombin aptamer was similar to that previously observed with PPACK and suggests that the thrombin aptamer can effectively inhibit thrombin and reduce platelet deposition at sites of vascular injury.

**DISCUSSION**

Using a novel selection and amplification technique, a new class of thrombin inhibitor based on ss oligodeoxynucleotides was recently discovered. In this study, using a synthetic 15-mer oligonucleotide (thrombin aptamer) that incorporates the consensus sequence, we showed that the thrombin aptamer effectively inhibited platelet aggregation in human platelet-rich plasma and FPA generation by fibrin-bound thrombin (Figs 1, 5, and 6). This was further substantiated in an ex vivo whole-artery angioplasty model in which the thrombin aptamer suppressed FPA generation and significantly reduced platelet deposition during high-shear conditions (Figs 7 and 8).

In this study, the aptamer’s antithrombin function was compared with two other inhibitors that inhibit thrombin by different mechanisms. Heparin, the most commonly used antithrombin agent, accelerates the irreversible inhibition of thrombin by its natural plasma inhibitor ATIII. PPACK is a synthetic peptide inhibitor that directly binds to the active site of thrombin, wherein the active site histidine is irreversibly alkylated by the chloromethyl ketone. In contrast to heparin, the thrombin aptamer does not interfere with thrombin’s amidolytic activity, indicating that it is not an active site inhibitor. Recent studies by ourselves and others suggest that the thrombin aptamer binds, in part, to the anion-binding exosite of thrombin and thereby interferes with thrombin’s interaction with fibrinogen and the platelet-surface thrombin receptor. The mode of interaction of the thrombin aptamer with thrombin is similar to that of a synthetic tyrosine-sulfated hirudin dodecapeptide (residues 53 to 64). However, the IC₅₀ for inhibition of platelet aggregation at 0.5 U/mL thrombin was reported to be 2.2 µg/mL (~1.7 µmol/L) for the hirudin peptide, which is substantially worse than that of the thrombin aptamer (~70 to 80 nmol/L) (Fig 2). Recently, we and others have obtained evidence showing that the thrombin aptamer has a discrete stable tertiary structure, which probably facilitates its interaction with thrombin.

Our data show that heparin at clinically relevant concentrations is effective in inhibiting free thrombin in plasma but is ineffective in inhibiting clot-bound thrombin activity (Fig 5). In an ex vivo whole-artery angioplasty model, even at a concentration of 2 U/mL, which is approximately 10 times its clinically achievable concentration, heparin was ineffective in suppressing FPA generation by thrombin bound on the denuded subendothelium (Fig 7). The resistance of clot-bound and matrix-bound thrombin to heparin-ATIII is in agreement with recent published studies and it is likely caused by steric hindrance and the neutralization of heparin by PPACK. Conversely, the effective inhibition of clot-bound and matrix-bound thrombin by the thrombin aptamer indicates that the anion-binding site of surface-bound thrombin is readily accessible to the ssDNA molecule. Recent studies suggest that the very high reocclusion rate observed after tPA administration in coronary thrombolysis may partially be caused by thrombolysis-induced coagulation activation. Successive removal of fibrin layers may lead to exposure of thrombin at the surface of the residual clot, which is functionally active and protected from heparin-ATIII. Our data show that the thrombin aptamer is superior to heparin in this setting and may have broad clinical applicability.

The thrombin aptamer-reduced platelet deposition on the balloon angioplasty denuded artery by approximately 60% at a shear rate of approximately 850 s⁻¹ (Fig 8). This degree of inhibition of platelet thrombus formation is comparable with that observed using high-dose PPACK. It should be noted that in this model that closely approximates clinical angioplasty, platelets are stimulated by multiple agonists such as ADP and collagen, in addition to thrombin, and thrombus formation is mediated by a variety of adhesion molecules. Using the monoclonal antibody 7E3, a potent platelet GPIIb-IIIa fibrinogen receptor antagonist, we obtained approximately 70% inhibition. Thus, the substantial but incomplete inhibition by the thrombin aptamer probably reflects the complex pathogenesis of arterial thrombosis, which is only partially dependent on thrombin and not caused by limited potency of the aptamer.

We have recently shown that the thrombin aptamer func-
bound thrombin is protected from inhibition by heparin-antithrombin III but is susceptible to inactivation by antithrombin III-independent inhibitors. J Clin Invest 86:385, 1990

Fig 8. Effect of thrombin aptamer on platelet deposition in an ex vivo whole-artery angioplasty model. Balloon-injured rabbit aortic segments were perfused, at both low (200 s⁻¹) and high (850 s⁻¹) shear rates, with heparinized (2 U/ml) human whole blood in the presence of thrombin aptamer (2 µmol/L, 2) or scrambled ssDNA control (2 µmol/L, 2). Platelet deposition of the denuded vessel after 30 minutes of perfusion was quantified using ¹²⁵I-labeled platelets. Results are standardized using platelet deposition in the presence of scrambled DNA as 100% (mean ± SEM, n=5).

Platelet Deposition (%)

Low

High

Shear

P=0.05

P=0.09

REFERENCES
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A novel nucleotide-based thrombin inhibitor inhibits clot-bound thrombin and reduces arterial platelet thrombus formation

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