Inhibition of Growth of Thrombus on Fresh Mural Thrombus
Targeting Optimal Therapy

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Background. Residual mural thrombus on severely damaged arterial wall is very thrombogenic. We tested the hypothesis that direct thrombin inhibition will block thrombus growth on fresh thrombus better than indirect thrombin inhibition, cyclooxygenase inhibition, or both.

Methods and Results. A fresh mural thrombus was formed by directly perfusing fresh porcine blood for 5 minutes over severely damaged arterial wall at a high shear rate in a well-characterized ex vivo perfusion system. The average platelet (P) and fibrinogen (F) deposition (D) achieved in 5 minutes were 382±32×10⁶ platelets/cm² and 296±36×10³ fibrinogen molecules/cm², respectively. Thrombus growth on the fresh mural thrombus was quantitated by directly perfusing blood from pigs with ¹²⁵I-labeled platelets and ¹³¹I-labeled fibrinogen for an additional 5 minutes over the preformed mural thrombus. Treatment included recombinant hirudin (1 mg/kg per hour IV) as a probe for thrombin, aspirin (5 mg/kg IV) as a platelet inhibitor of cyclooxygenase, heparin (moderate, 100 IU/kg per hour IV; high-dose, 250 IU/kg per hour IV) as an indirect thrombin inhibitor, and heparin (100 IU/kg per hour) plus aspirin (5 mg/kg IV). Thrombus growth as measured by labeled PD (×10⁶/cm²) and FD (×10¹² molecules/cm²) was mildly but not significantly reduced by aspirin (1034±92 and 436±78, respectively) compared with baseline (1113±67 and 545±52, respectively). Inhibition of thrombus growth with heparin was dose dependent. A regression analysis showed an inverse correlation of PD and FD with mean plasma heparin concentrations (r=−.81, P=.0001 and r=−.49, P=.0007, respectively). Recombinant hirudin led to a profound inhibition of thrombus growth (PD, 30±12; FD, 109±21), which was significant compared with all groups, even the highest dosage of heparin (250 IU/kg per hour).

Conclusions. Specific thrombin inhibition markedly inhibits platelet and fibrinogen deposition onto fresh mural thrombus at a high shear rate. Aspirin alone or in combination with heparin has little effect on evolving thrombosis. Heparin dose dependently reduces thrombus growth, but even the highest dosage is less effective than hirudin. Thrombin appears to be the primary activator of platelets by fresh thrombus.

Key Words: thrombosis • atherosclerosis • reocclusion • antithrombotics • hirudin

Platelet-rich thrombus formation on a disrupted atherosclerotic plaque is an important pathogenetic factor in the development of acute coronary syndromes.¹⁶ Thrombin generation and platelet aggregation play key roles in the progression of coronary thrombosis.³² The goal of treatment in unstable angina and after thrombolysis for acute myocardial infarction is to block growth of thrombus and to prevent new occlusions.

Angiographic studies,⁸,⁹ stressed the importance of residual mural thrombus for predisposition to rethrombosis after thrombolysis. Factors that may contribute to rethrombosis include reexposure of damaged vessel wall,¹⁰ thrombin bound to clot or arterial wall matrix,¹¹,¹² platelet secretory products,¹³ and thrombolysis-induced platelet activation or stimulation of thrombin generation.¹⁴ Failure of medical therapy in acute coronary syndromes most often occurs within the first days of treatment.¹⁵ Increased thrombin generation¹⁶ may persist beyond the acute event of thrombolytic treatment and lead to late reocclusions despite current antithrombotic treatment.¹⁷,¹⁸

We used our porcine ex vivo perfusion model¹⁹ to study the growth of arterial thrombus onto preformed mural thrombus under controlled rheologic conditions corresponding to a moderate coronary stenosis. We hypothesized that specific direct antithrombin therapy with hirudin would inhibit growth of thrombus onto fresh thrombus better than indirect antithrombin or cyclooxygenase inhibition with current medical therapies (aspirin, heparin, or both). Our results suggest that specific thrombin inhibition profoundly blocks the growth of thrombus on fresh arterial thrombus.

Methods

To quantitatively study the growth of arterial thrombus onto preformed fresh thrombus induced by severely injured arterial wall, we modified our porcine ex vivo perfusion system²⁰–²¹ as described below using fresh blood to measure ¹²⁵I-labeled platelets and ¹³¹I-labeled fibrinogen depositions onto severely injured arterial wall (mural thrombus) and onto preformed unlabeled mural thrombus (growth of thrombus).

Animal Preparation

All procedures performed in this study were approved by the institutional guidelines of the Animal Subjects Committee.
of Harvard University and followed the American Heart Association guidelines for animal research. Yorkshire albino pigs were obtained from the same colony of pigs (n = 14) from a single local farmer (body weight, 29 ± 2.3 kg).

The day before the experiment, blood was drawn from the pigs via the femoral artery. Platelets were labeled with \(^{111} \text{In}\) tropolone and fibrinogen was labeled with \(^{125} \text{I} \) as previously described.\(^{22,23}\) Porcine fibrinogen was isolated from the same breed of pigs used for the perfusion experiments according to the \(\beta\)-alanine technique.\(^{24}\)

Eighteen to 24 hours after labeling autologous platelets with \(^{111} \text{In}\), the pigs were sedated with ketamine (20 mg/kg IM) and atropine (0.05 mg/kg IM), anesthetized with sodium pentobarbital (10 mg/kg IV), and intubated and ventilated (Harvard respirator). Anesthesia was maintained by repeat intravenous bolus of sodium pentobarbital with the minimal effective dose as previously described (4 to 6 mg/kg IV).\(^{23}\) The carotid artery and the contralateral external jugular vein were cannulated through a longitudinal left and right neck incision to establish an extracorporeal circuit as previously described.\(^{25,26}\) The carotid artery was directly connected by polyethylene tubing (20 cm long; PE 200, Clay Adams, Division of Becton, Dickinson and Co) to the input of the Plexiglas chamber. The output of the chamber was connected to a peristaltic pump (model 7013, Masterflex, Cole-Palmer Instrument Co). Blood that had passed through the perfusion chamber was recirculated back into the animal by the contralateral external jugular vein. All animals received low-dose anticoagulation with heparin (50 IU/kg; mean activated partial thromboplastin time [aPTT] ratio, 1.4 ± 0.1) as a continuous infusion to avoid clotting inside the tubing system. Low-dose heparin does not affect platelet or fibrinogen deposition compared with native (nonanticoagulated) blood.\(^{25,26}\)

**Perfusion Chamber**

We used our previously characterized perfusion chamber, which mimics the cylindrical shape of the blood vessels.\(^{19,21,23}\) The substrate was placed in the lateral wall of the lumen of the blood channel of the perfusion chamber and was directly exposed to fresh blood as described below. In the present study, we used a chamber with an internal diameter of 0.1 cm modeling local flow conditions typical of medium-grade stenosis in coronary arteries (Reynolds’ number [Re] = 60; average velocity \(V_v\) = 21 cm/s, shear rate [SR] = 690 s\(^{-1}\)). At these flow conditions, blood can be considered as having newtonian fluid properties with constant viscosity. Shear conditions at the vessel wall were calculated from the theoretical expression for shear rate given for a newtonian fluid in tube flow.\(^{27}\)

**Preparation of Vessel Segments**

Pig tunica media prepared from aortas harvested from healthy untreated pigs were used as a model of severe arterial wall damage.\(^{28}\) The aortas were obtained fresh, cleaned of the surrounding connective tissue, immediately deep-frozen in liquid nitrogen, and stored at -70°C. Before the experiments, the aortas were thawed in phosphate-buffered saline (PBS) at 4°C, opened longitudinally, and cut into 25 x 10-mm segments. The intima with a thin portion of the subjacent media was removed, leaving the substrate with the major portion of the remaining media.

**Perfusion Conditions**

Tunica media vessel wall segments were mounted in the chamber and perfused with PBS solution in a water bath at 37°C for 60 seconds. Then, blood was perfused through the chamber for a selected period of time, followed by perfusion with buffer for 60 seconds to clear away unattached cells and blood.

**Quantitation of Thrombus Growth**

To quantitate growth of thrombus on fresh thrombus formed on tunica media, we modified the previously described perfusion conditions\(^{21}\) by using blood from two pigs in the same experiment. One pig with unlabeled blood was used to form a fresh thrombus. The second pig with \(^{125} \text{I}\)-labeled platelets and \(^{125} \text{I}-\)labeled fibrinogen was used to quantitate the growth of thrombus under identical flow conditions (Fig 1).

After the preperfusion period with buffer, unlabeled blood from pig A was passed through the chamber at a flow rate of 10 ml/min for 5 minutes to create a fresh thrombus (formed on severely injured arterial wall). Then, the growth of thrombus on fresh (unlabeled) thrombus as a substrate was measured using blood from pig B containing autologous \(^{111} \text{In}\)-labeled platelets and \(^{125} \text{I}\)-labeled fibrinogen under the same flow conditions for 5 minutes (thrombus growth, Fig 1). The changes from buffer to blood A, blood A to blood B, and blood B to buffer were achieved manually by three-way stop cocks without introducing stasis or air in the chamber. One stop cock was used to discard blood and buffer before autologous blood was recirculated through the jugular vein.

**Evaluation of the Perfusion Model of Thrombus Growth**

We have previously shown that the rate of platelet deposition on severely damaged vascular wall at high shear rates is characterized by a first-order rate constant.\(^{29}\) To evaluate the
growth of thrombus on a preformed thrombus, it was important to know whether the growth rate of platelet and fibrin deposition was identical when using blood from two different animals containing unlabeled (pig A) and 121I-labeled (pig B) platelets and 125I-fibrinogen.

Experiments were carried out to evaluate the amount of fresh thrombus (5-minute perfusion with labeled blood, pig B), the amount of thrombus growth (5-minute perfusion of labeled blood [pig B] on an unlabeled fresh thrombus [pig A], as described above), and the total platelet and fibrinogen deposition over 10 minutes (10-minute perfusion, pig B, Fig 1). The sum of both amounts of fresh thrombus and thrombus growth was compared with the amount of platelet and fibrinogen deposition following the 10-minute perfusion and was found to be similar.

Experimental Protocol
In all experiments, baseline perfusions were performed to evaluate the amount of fresh thrombus (n=4) and thrombus growth (n=4). Subsequently, pig B with 11In-labeled platelets and 125I-labeled fibrinogen was given the selected treatment regimen, and perfusions were continued (n=6) to measure the effect of treatment on growth of thrombus on fresh thrombus. Perfusion experiments of each treatment group were carried out in duplicate.

The first group received aspirin at a dose of 5 mg/kg by intravenous injection (lysinated acetylsalicylic acid; Synthelabo-Pharma). Arachidonic acid-induced platelet aggregation in whole blood was measured 20 minutes later and was completely abolished. The second group was given heparin as an intravenous bolus of 100 IU/kg per hour during the treatment period (heparin sodium, USP, derived from porcine intestinal mucosa; Elkins-Sinn, Inc). The third group received the same dose of heparin in combination with intravenous aspirin (5 mg/kg). The fourth group was given heparin as an intravenous bolus of 250 IU/kg followed by a continuous infusion of 250 IU/kg per hour. The fifth group received hirudin (recombiant desulfato hirudin; CPG 39393, CIBA-GEIGY) as an intravenous bolus of 1 mg/kg followed by a continuous infusion of 1 mg/kg per hour. This hirudin concentration totally inhibited thrombin-induced platelet aggregation in whole blood.

Laboratory Measurements
After each sequence of four perfusions, blood samples were collected from each pig and evaluated for platelet count, indium lysis, fibrinogen levels, hematocrit, prothrombin time, aPTT, thrombin time (TT), and heparin levels. Heparin levels were measured according to the level of anti-factor Xa coagulation activity (assay kit, Diagnostica Stago).

Radioactive Labeling of Platelets and Fibrinogen
Twenty-four hours before the perfusion experiments, autologous platelets were labeled with 111In-(tropolone) as previously described.19,22 The final pellet of labeled platelets suspended in 4 mL of platelet-poor plasma (2.6±0.9×10^10/mL) was reinfused into the animal after low-spin centrifugation to remove any microaggregates. The average labeling efficiency was 55±8%. The injected activity was 243±54 μCi.

Porcine fibrinogen was labeled with 125I (Du Pont NEN) using the Iodogen technique30 (Iodogen, Pierce). The amount of radioactivity bound to the protein, estimated by trichloroacetic precipitation, was more than 95%, and the percent of elutable protein was 99%. An average of 270±77 μCi was used for each labeled pig.

Quantitation of Platelet and Fibrinogen Deposition
The number of platelets (×10^10) and the molecules of fibrinogen (×10^12), some incorporated into fibrinogen, deposited on each substrate were quantified by the method of Dewanjee et al.31 Counting for 111In-labeled platelet and 125I-labeled fibrinogen deposition on the perfused substrates were determined by gamma-well counter (Packard Auto-Gamma 5650) using two windows of 150 to 490 KeV and 15 to 80 KeV, respectively. The number of platelets deposited on the perfused matrix was determined by its 111In cpm counts, normalized by blood activity (counts), blood platelet counts, and surface area.23 The fibrinogen 125I cpm counts of the substrates were normalized by blood activity, fibrinogen plasma levels, and surface area.41

Statistical Analysis
Statistical comparison of data was carried out using StatView II (Abacus Concepts Inc). Between-group analysis were made using one-way ANOVA, followed by Fisher PLSD and Scheffe F test to assess specific group differences. Log of number of platelets and molecules of fibrinogen was regressed against aPTT and plasma heparin concentrations with linear regression. All values are presented as mean±SEM unless otherwise stated. P<.05 was considered significant.

Results
Model of Thrombus Progression
We have developed a perfusion model of arterial thrombosis for the study of growth of thrombus on fresh mural thrombus, which is preformed on severely injured arterial wall (Fig 2). All animals received low-dose anticoagulation with heparin to avoid clotting inside the tubing system. Deposition of fresh thrombus was measured by perfusing porcine aortic tunica media for 5 minutes with arterial blood directly from pigs with 111In-labeled platelets and 125I-labeled fibrinogen. A mean of 401±93×10^9 platelets/cm^2 and 296±36×10^12 fibrinogen molecules/cm^2 were deposited on the vascular substrates as fresh thrombus.

An unlabeled fresh thrombus was created by perfusing the vascular substrate with unlabeled blood for 5 minutes under the same flow conditions. Then, this unlabeled fresh thrombus was perfused with blood from a pig with 111In-labeled platelets and 125I-labeled fibrinogen for an additional 5 minutes. A mean of 904±221×10^9 platelets/cm^2 and 546±52×10^12 fibrinogen molecules/cm^2 were deposited on the preformed unlabeled thrombus. The sum of the amount of fresh thrombus and new growth of thrombus as measured by 111In-labeled platelet and 125I-labeled fibrinogen deposition for successive 5-minute intervals were equal to the amount of 111In-labeled platelet and 125I-labeled fibrinogen deposition during a single perfusion for 10 minutes (1343±364×10^9 platelets/cm^2 and 810±101×10^12 fibrinogen molecules/cm^2), emphasizing the reproducibility of our model of thrombus progression (Fig 2A and 2B).

Effect of Treatment Regimens on Thrombus Progression
The growth of thrombus on preformed fresh thrombus (n=32) and the effect of various treatments on the growth of thrombus (n=12 each) are shown in Fig 3A and 3B.

The mean deposition of 111In-labeled platelets and 125I-labeled fibrinogen molecules on a fresh thrombus at high shear rate in pigs treated with aspirin compared with baseline was not significantly different.

Platelet and fibrinogen depositions in those treated with heparin 100 IU/kg per hour and the combination of heparin 100 IU/kg per hour and aspirin were moderately and significantly lower compared with baseline but
not significantly different from each other. A further reduction of platelet and fibrinogen deposition in pigs treated with very high doses of heparin (250 IU/kg bolus plus 250 IU/kg per hour infusion) occurred when compared with baseline (P<.001 and <.02, respectively) and compared with those treated with lower doses of heparin (100 IU/kg per hour, P<.01 and =.13, respectively). In fact, log of platelet deposition showed a strong and significant inverse relation with aPTT levels (r = -.80, P = .0001) and mean plasma heparin concentrations (r = -.81, P = .0001; Fig 4A). Similarly, molecules of fibrinogen deposited were inversely correlated with aPTT (r = -.44, P = .003) and mean plasma heparin concentrations (r = -.49, P = .0007; Fig 4B).

Hirudin significantly reduced platelet and fibrinogen deposition, even compared with the highest concentration of heparin (P<.001 and <.001, respectively).

To assess the level of anticoagulation in the different experimental groups in this study, we performed the aPTT test on all pigs. Heparin levels were also directly measured using anti-factor Xa activity test. Table 1 presents mean aPTT values and heparin levels obtained from hourly samples taken over a 6-hour period. At similar aPTT levels, hirudin was more effective in reducing platelet and fibrinogen deposition on a fresh thrombus than heparin. Note that baseline perfusions of all experimental groups were carried out at a low level of anticoagulation (mean aPTT ratio, <1.5 with 50 IU/kg heparin).

**Laboratory Data**

Platelet count, fibrinogen levels, and hematocrit were assessed every hour during baseline perfusions and during the treatment period. There was a slight nonsignificant reduction in platelet count from 447±18 to 421±15×10⁴/μL during the treatment phase compared with the baseline perfusion period. Fibrinogen levels and hematocrit remained unchanged from 259±23 to 253±21 mg/dL and from 31±0.8% to 30±1.0%, respectively. Platelet counts, fibrinogen levels, and hematocrit did not differ significantly between treatment groups.

**Discussion**

This study demonstrates the primary thrombin dependence of growth of thrombus as shown by the therapeutic probe hirudin in blocking growth of thrombus. This is consistent with the increased thrombin content of thrombus adjacent to severely injured arterial wall, the increased fibrinogen/fibrin deposition in the layers of thrombus immediately adjacent to severely injured arterial wall, the binding of active thrombin to fibrin and arterial wall matrix, and the masking of receptors to antithrombin III and heparin cofactor II on thrombin bound to fibrin.

This study supports the hypothesis that platelet and fibrinogen depositions onto fresh mural thrombus at high shear rate are primarily thrombin dependent and decrease with increasing antithrombin activity. Platelet and fibrinogen depositions induced by fresh mural thrombus were lowest in pigs treated with the specific thrombin inhibitor hirudin, which does not inhibit other platelet activators but completely abolished growth of thrombus. The antithrombin effect of heparin was incomplete at usual therapeutic doses but improved at very high doses. Recombinant hirudin (r-hirudin) was more effective than high-dose heparin at lower aPTT levels. The effect of aspirin (alone or combined with
heparin) on thrombus growth at high shear was mild and not significant.

In acute coronary syndromes and after reperfusion, a fresh mural thrombus over atherosclerotic plaque may contribute to residual stenosis and stimulate thrombus growth to total occlusion. This ex vivo perfusion model in the pig is useful for quantitating the growth of thrombus onto a preformed thrombus and for testing the efficacy of various antithrombotic treatments in blocking growth of thrombus.

Thrombus formation on areas of severely injured artery is greatly dependent on local thrombin production at the site of vascular damage. Specific thrombin inhibition with hirudin was more effective than high-dose heparin in preventing mural thrombosis in vivo and ex vivo onto severely injured artery in pigs. In this study, growth of thrombus triggered by a fresh thrombus was also primarily thrombin dependent and effectively inhibited by specific thrombin inhibition at a dose that successfully prevented mural thrombosis after severe arterial injury by balloon dilation in pigs.

The superior antithrombotic efficacy of specific thrombin inhibition appears to be due to several mech-
to be primarily thrombin mediated. Specific thrombin inhibition with hirudin has a dramatic inhibitory effect on platelet and fibrinogen deposition during growth of mural thrombus and may be a superior treatment strategy for acute coronary syndromes and prevention of reocclusion after thrombolytic therapy. Future studies need to address the optimal duration of antithrombin therapy.

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