Basic Science Reports

Dissolution of Mural Thrombus by Specific Thrombin Inhibition With r-Hirudin
Comparison With Heparin and Aspirin

Beat J. Meyer, MD; Juan J. Badimon, PhD; James H. Chesebro, MD; John T. Fallon, MD; Valentin Fuster, MD, PhD; Lina Badimon, PhD

Background—The presence of residual mural thrombus may predispose to recurrent thrombotic events in acute coronary syndromes. The purpose of this study was to evaluate the effects of antithrombotic and antiplatelet agents on a preformed, fresh mural thrombus during growth of thrombus.

Methods and Results—A fresh mural thrombus was formed by perfusing severely injured arterial wall with porcine blood for 5 minutes at a shear rate of 1690 s⁻¹. Thrombus formation was measured by morphometric analysis (mm²/mm). The average size of a mural thrombus formed in 5 minutes was 0.14±0.03 mm²/mm. Progression of thrombus growth within 10 minutes triggered by the preformed thrombus was evaluated in pigs treated with r-hirudin (1 mg/kg per hour IV) as a probe for thrombin, high-dose heparin (250 IU/kg per hour IV), moderate-dose heparin (100 IU/kg per hour), moderate-dose heparin (100 IU/kg per hour) plus aspirin, aspirin alone (5 mg/kg IV), and placebo. Hirudin was associated with a significant decrease (48%) of mural thrombus area and significantly reduced growth of thrombus (0.07±0.01), even compared with the highest dose of heparin (0.15±0.03), although at lower levels of anticoagulation. Inhibition of growth of thrombus with heparin was dose dependent, showing an inverse correlation of thrombus area with mean plasma heparin concentrations (r=-.77, P=.0001). Thrombus size was unchanged by aspirin (0.29±0.07) compared with controls (0.28±0.07).

Conclusions—Direct inhibition of thrombin activity with r-hirudin completely inhibits growth of thrombus, causes dissolution of a preexisting mural thrombus, and is more effective at lower levels of anticoagulation than the highest dose of heparin at shear rates typical of a moderate coronary stenosis. (Circulation. 1998;97:681-685.)

Key Words: thrombus ■ hirudin ■ thrombosis ■ platelet aggregation inhibitors

Thrombin plays a pivotal role in response to rupture of an atherosclerotic plaque, leading to coronary thrombosis in patients with acute coronary syndromes.¹ The major effects of this serine protease include promotion of fibrin formation and further activation of the coagulation cascade by prothrombinase complex. In addition, thrombin is a potent stimulus for platelet activation, induction of adhesion molecules by neutrophiles and monocytes, and proliferation of vascular smooth muscle cells.²,³ Thrombus formation is modulated by local, rheological, and substrate factors and systemic factors. Recent angiographic and experimental studies demonstrated that a residual mural thrombus is a highly thrombogenic surface.⁴ Residual mural thrombus and persistent thrombin generation may predispose to recurrent thrombotic events and reocclusion after thrombolysis.⁶⁻¹⁰

The current approach of combining antiplatelet and antithrombotic therapy is based on the proposed roles of both activated platelets and thrombin in growth of arterial thrombus in acute coronary syndromes. Despite the proven clinical efficacy of heparin and aspirin in the treatment of acute coronary syndromes, more effective regimens with acceptable safety profiles are needed.

Several groups have studied the relationship among surface composition, shear rate, and platelet thrombus formation, but very few have studied the effect of an already formed platelet-rich thrombus on the growth of thrombus under flow conditions similar to a moderately stenosed coronary artery. In a previous study, we used an extracorporeal perfusion model to study the growth of thrombus triggered by a fresh mural thrombus.⁷ Growth rate was measured using blood containing ¹¹¹In-labeled platelets and ¹²⁵I-labeled fibrinogen. In this study, we analyzed the perfused aortic segments histologically and applied morphometric analysis to quantify the effects of different antithrombotic treatments on the preformed mural thrombus. We tested the hypothesis that thrombin activity is necessary for cohesion of platelet-rich thrombus and that specific thrombin inhibition but not current antithrombotic therapy is effective in deaggregating fresh platelet-rich mural thrombus.

Methods

Animal Preparation

All procedures performed in this study were approved by the institutional guidelines of the Animal Committee and conformed with...
Dissolution of Mural Thrombus

the American Heart Association guideline for animal research. Yorkshire albino pigs were obtained from the same colony of pigs (n=14) from a single local farmer (body weight, 29±3 kg). As described previously, 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, Harvard Apparatus). Anesthesia was maintained by repeat intravenous boli of sodium pentobarbital with the minimal effective dose as previously described (4 to 6 mg/kg IV).11

The carotid artery and contralateral external jugular vein were cannulated through a longitudinal left and right neck incision to establish an extracorporeal circuit as previously described.12 The carotid artery was directly connected with polyethylene tubing (20 cm in length; Clay Adams, PE 200, Division of Becton, Dickinson and Co) to the input of the Plexiglas chamber. The output of the chamber was connected to a peristaltic pump (Masterflex, model 7013; Cole-Palmer Instrument). 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 fibrin(ogen) deposition compared with native (nonanticoagulated) blood.13,14

Perfusion Chamber

We used our previously characterized perfusion chamber, which mimics the cylindrical shape of the blood vessels.12,13,15 The substrate was placed on the wall of the blood channel of the perfusion chamber and directly exposed to fresh flowing blood as described below. We used a chamber with an internal diameter of 0.1 cm, modeling local flow conditions typical of medium-grade stenosis in coronary arteries (shear rate, 1690 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.16

Evaluation of Thrombus Growth

Tunica media prepared from pig aortas was used as a model of severe arterial wall damage.17 To quantify growth of thrombus on fresh thrombus formed on tunica media, we modified the previously described perfusion conditions by using blood from two pigs in the same experiment.18 One pig served as a blood donor to form a fresh mural thrombus. The second pig was treated with different antithrombotic treatment regimens and used to quantify the growth of thrombus under identical flow conditions.

After the preperfusion period with buffer, blood from the first pig was passed through the chamber at a flow rate of 10 mL/min for 5 minutes to create a fresh thrombus on tunica media. Then, the growth of thrombus on preformed fresh thrombus was measured under the same flow conditions using blood from the second pig assigned to different antithrombotic treatment groups. The changes of perfusion from buffer to blood, from blood of the first pig to blood of the second pig, and from blood to buffer were achieved manually with three-way stopcocks without the introduction of stasis or air in the chamber. One stopcock was used to discard blood and buffer before autologous blood was recirculated through the jugular vein.

This method of evaluation of growth of thrombus on a preformed thrombus has been validated previously.18 We have shown that the rate of platelet deposition on severely damaged wall at high shear rates is characterized by a first-order rate constant.18

Experimental Groups

The experimental groups were the same as in our previous study.7 In brief, baseline perfusions were performed to evaluate the amount of fresh thrombus (baseline, n=8) and thrombus growth (control, n=8) in all experiments. Subsequently, the second animal was given the selected treatment regimen, and perfusions were continued for each group (n=12) to measure the effect of treatment on fresh mural thrombus and growth of thrombus.

The first group received aspirin at a dose of 5 mg/kg IV (fumaryl acetylsalicylic acid; Synthelabo-Pharma). This dose of aspirin completely abolished arachidonic acid–induced platelet aggregation (0.9 mmol/L final concentration; Sigma Chemical) in whole blood measured 20 minutes later. The second group was administered heparin as an intravenous bolus of 100 IU/kg followed by a continuous infusion of 100 IU/kg per hour during the treatment period (heparin sodium USP, derived from porcine intestinal mucosa; Elkins-Sinn). 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 (recombinant desulfatidillaudin; CGP 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 (5 U/mL; Sigma) in whole blood.

Morphometric Quantification of Thrombus Formation

Radiolabeled platelets and fibrinogen are useful in studying the rate of thrombus growth, as shown in our previous study.7 However, the morphometric analysis used in this study allowed evaluation and characterization of the total thrombus area, including the preformed fresh mural thrombus. After the perfusion, the aortic media strips were removed from the chamber, fixed in 4% paraformaldehyde, dehydrated with graded alcohol series, embedded in paraffin, and sectioned. From each vessel, six sections (4 μm thick) were taken at 100-μm intervals parallel to the direction of flow. The sections were stained with hematoxylin and eosin and trichrome. The single section with the greatest amount of thrombus, coinciding with the longitudinal central line of the surface exposed to blood, was systematically chosen from each vessel for morphometric evaluation. Thrombus size was quantified morphometrically by viewing the thrombus mass through the microscope at 100× magnification and tracing the outline using a side-tube attachment to the microscope. The traced outline was then scanned into a Macintosh computer, and areas were calculated using an image processing software (NIH Image 1.44 for Macintosh). The thrombus size was expressed as the surface area (mm2) normalized to the length of the exposed segment (mm). Morphometric methods have been previously validated for this model and other models and show a strong correlation between the amount of 111In-labeled platelets deposited on the media and the morphometrically assessed thrombus size.18-21 All measurements were made in a blinded fashion.

Laboratory Measurements

After each sequence of four perfusions, blood samples were collected from each pig and evaluated for platelet count, hematocrit, aPTT, and heparin levels. Heparin levels were analyzed by an assay using a chromogenic substrate. The test is based on the in vitro anti–factor Xa activity. The release of pNA of the chromogenic substrate is inversely proportional to the amount of heparin (IU/mL) present in the plasma (Stachrom assay, Diagnostica Stago). The heparin standard used in our heparin assays included three levels of UFH calibrators (Heparinom H; Diagnostica Stago).

Data Analysis

Statistical comparison of data were carried out using StatView II (Abacus Concepts). Between-group analysis were made using one-way ANOVA, followed by Fisher PLSD and Scheffe’s F test to assess specific group differences. Thrombus area 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

Thrombus Formation on Fresh Mural Thrombus

Mural thrombus was formed on an arterial media as illustrated in Fig 1. The total area of thrombus formed on preformed fresh thrombus (control, n=32) and the effect of various antithrom-
botic agents on growth of thrombus (n=12 each) are presented in Fig 2. Fresh mural thrombus induced growth of thrombus by doubling of measured thrombus size in untreated groups (0.14±0.03 and 0.28±0.07 mm²/mm, respectively).

Hirudin was associated with a significant decrease of total thrombus area compared with baseline mural thrombus formation, indicating dissolution of preformed mural thrombus (P<.001, Fig 1). Direct thrombin inhibition by hirudin significantly reduced growth of thrombus compared with control and even compared with the highest concentration of heparin (P<.001 and P<.001, respectively; Fig 2). Thrombus size in groups treated with heparin 100 IU/kg per hour alone and the combination of heparin 100 IU/kg per hour and aspirin was significantly lower compared with controls but was not significantly different from each other. A greater inhibition of growth of thrombus in pigs treated with very high doses of heparin (250 IU/kg bolus plus 250 IU/kg per hour infusion) occurred when compared with control (P<.001) and compared with those treated with lower doses of heparin (100 IU/kg per hour, P<.01). In fact, thrombus size correlated strongly and significantly with plasma heparin concentrations (r=.77, P=.0001, Fig 3) and aPTT levels (r=.65, P=.0001).

Thrombus size was not changed in pigs treated with aspirin alone compared with controls.

Hemostatic Parameters
Platelet count 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±15×10^3/μL during the treatment phase compared with the baseline perfusion period. The hematocrit remained unchanged from 31±0.8% to 30±1.0%. Platelet counts and hematocrit were not significantly different between treatment groups.

To assess the level of anticoagulation in the different experimental groups in this study, the aPTT test and heparin levels using anti-factor Xa activity test were performed from hourly samples taken over a 6-hour period. Note that baseline and control perfusions of all experimental groups were carried out at a low level of anticoagulation (mean aPTT ratio, <1.5 with 50 IU/kg heparin). In the heparin (100 IU) and heparin (100 IU)-plus-aspirin groups, the aPTT was prolonged to 2.4±0.26 and 2.2±0.03 times baseline control value, respectively. In the heparin (250 IU) group, the aPTT was prolonged to >12.1 times baseline control value. In the hirudin group, the aPTT was prolonged to 3.3±0.01.

Discussion
The present study revealed that specific thrombin inhibition with r-hirudin induced dissolution of preformed mural thrombus in an experimental model of deep injury and platelet-rich thrombosis. This observation of dissolution of fresh mural thrombus by specific thrombin inhibition alone without concomitant administration of a fibrinolytic agent represents a substantial extension of our previous findings9 and underscores the importance of thrombin in cohesion of fresh platelet thrombus.

Specific thrombin inhibition not only completely blocked growth of thrombus but also significantly decreased thrombus size of preformed mural thrombus at lower levels of anticoagulation than those achieved with high-dose heparin. Growth of thrombus induced by preformed, fresh mural thrombus was lowest in pigs treated with the specific thrombin inhibitor r-hirudin at a dose that prevented macroscopic mural thrombus after deep injury13,14 and increased with decreasing anti-thrombin activity, confirming our previous results.9,15,16 Thrombus size in animals treated with aspirin was not significantly reduced at these rheological conditions, a finding that is consistent with previous results.9,22

Thrombin plays a central role in the formation, growth, maintenance, and consolidation of thrombus. Direct thrombin inhibition with hirudin, but not heparin, blocks these processes and leads to profound inhibition of thrombus14,23–25 and even to dissolution of preformed thrombus, as in our study. In vivo, in the pig with a carotid artery crush injury, 90% of the preformed, half-hour-old thrombus dissolved within 1 hour of hirudin administration.26 The superior antithrombotic efficacy of specific thrombin inhibition over heparin appears to be due to several mechanisms, including better inhibition of catalytically active clot-bound thrombin,27,28 lack of natural inhibitors against hirudin but present against heparin,29,30 and high affinity for platelet thrombin receptor by r-hirudin, which can displace
thrombin bound to platelet receptor. Antithrombins with greater binding affinity to thrombin have greater antithrombotic potential, as demonstrated by graded reduction in thrombin affinity of r-hirudin mutants, which resulted in a progressive attenuation of antiplatelet activity.

In addition, the significant decrease of thrombus size seen in our study with specific thrombin inhibition suggests disaggregation of platelets, facilitated endogenous fibrinolysis, or both, an observation also made in recent in vitro and in vivo studies. Most likely, disaggregation of platelet-rich thrombi is the predominant mechanism of dissolution of the mural thrombi in the present study. Although stable thrombin inhibition may enhance endogenous fibrinolysis, the mechanism by which this effect is obtained remains unclear. Inhibition of platelet aggregate formation and thrombin-mediated fibrin formation appears to shift the dynamic hemostatic balance toward fibrinolysis, resulting in an overall reduction in thrombus size.

Contributing mechanisms for facilitating endogenous fibrinolysis may be the inhibition of fibrin cross-linking, which is necessary for stable clot formation; cross-linking of α2-antiplasmin to fibrin monomers, which is dependent on the activation of factor XIII by thrombin, seems to be essential for thrombolysis resistance of fibrin clots. In addition, specific thrombin inhibition may facilitate endogenous fibrinolysis by lowering PAI-1 secretion of endothelial cells and inhibiting the release of PAI-1 from platelets.

In the present study, dissolution of platelet-rich thrombus occurred at a dose of r-hirudin that completely abolished platelet thrombus formation in previous studies. In patients receiving r-hirudin for 3 to 5 days, thrombus dissolution in aortocoronary vein grafts occurred at aPTT levels that were two to three times those of control. However, if r-hirudin is administered in combination with thrombolytic agents (rt-PA), residual platelet-rich thrombus may be eliminated at lower levels of anticoagulation (aPTT ratios, 2.5 times control). In experimental studies of coronary thrombosis, hirudin was superior to heparin in facilitating thrombolysis with both t-PA and streptokinase and almost completely eliminated residual platelet-rich thrombus. Dissolution of mural thrombus by specific thrombin inhibition with r-hirudin is of potential therapeutic benefit after acute coronary syndromes that could lead to improved vessel patency with reduced stenosis, thus decreasing the need for revascularization procedures in a significant proportion of patients.

In summary, specific thrombin inhibition with r-hirudin blocks the growth of thrombus and leads to a reduction of the mural thrombus during the growth of thrombus, indicating reversibility of a preexisting mural thrombus. It appears that continued cohesion of fresh platelet-rich mural thrombus is thrombin dependent. r-Hirudin has a superior antithrombotic effect com-

Figure 2. A, Differential effects of antithrombotic regimens on mean thrombus area during growth of thrombus (control, hatched bar) triggered by a fresh mural thrombus at high shear rate (baseline, open bar). Vertical lines, SEM. Thrombus area in animals treated with hirudin was significantly lower compared with baseline and the highest heparin group (*P < .001 versus baseline, §P < .001 versus heparin 250 IU/kg). Heparin showed a dose-dependent effect on growth of thrombus. Aspirin had no significant effects on growth of thrombus. B, Growth of thrombus as a percentage of mural thrombus of the same experimental groups in A. Note a significant reduction of mural thrombus during treatment with r-hirudin, indicating dissolution of preformed mural thrombus.

Figure 3. Relation between thrombus area and heparin levels (IU/mL) during growth of thrombus onto a mural thrombus in pigs treated with heparin doses of 50, 100, or 250 IU/kg. The significant inverse relations is shown. Two values of heparin levels in the group receiving 50 U heparin are missing; one value is hidden due to overlapping results in the top left cluster.
pared with heparin at lower levels of aPTT. Heparin exerts a dose-dependent inhibition of growth of thrombus induced by a fresh mural thrombus. Aspirin alone or as an additional drug to heparin has little effect on the progression of a thrombus at high shear rates. Therefore, specific thrombin inhibition appears to remain a promising treatment, but further studies are required to substantiate these potential benefits in humans.

Acknowledgments
This work was supported in part by NIH grant HL-38933 and CICYT grant SAF-94/0712. Dr Meyer is supported by grant 32–47077.96 from the Swiss National Foundation of Science.

References
Dissolution of Mural Thrombus by Specific Thrombin Inhibition With r-Hirudin: Comparison With Heparin and Aspirin
Beat J. Meyer, Juan J. Badimon, James H. Chesebro, John T. Fallon, Valentin Fuster and Lina Badimon

_Circulation_. 1998;97:681-685
doi: 10.1161/01.CIR.97.7.681
_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1998 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/97/7/681

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation_ is online at:
http://circ.ahajournals.org//subscriptions/