The role of thrombin inhibition in platelet vessel wall interaction and thrombus growth was studied under controlled flow conditions. Natural hirudin and recombinant hirudin (r-hirudin), which are specific thrombin inhibitors, were compared with heparinized blood (1.8 ± 0.2 U/mL) and Ca²⁺-chelated blood in their potential to inhibit platelet interaction and thrombus growth on two biologic vascular surfaces and one immobilized vessel wall component. The substrates were perfused by flowing blood at shear rates typical of patent and stenosed arteries (212 to 1,690/s) for 5 minutes. Platelet deposition was measured by in-111-labeled platelets. We found that both natural and r-hirudin have similar effects on platelet-substrate interaction. As compared with heparin, platelet deposition to mildly damaged vessel wall and digested collagen type I was not reduced by hirudin or citrate. However, hirudin and citrate significantly reduced platelet deposition to severely damaged vessel wall (platelets x10⁶/cm²: 93 ± 10 in heparinized blood vs 60 ± 7 in blood treated with 100 U/mL r-hirudin). Therefore, thrombus growth on areas of severe wall damage is in part dependent on local thrombin production at the site of vascular damage. We also found that hirudin added to heparinized blood reduced platelet deposition to severely injured wall but not to subendothelium or collagen-coated slides. Hirudin added to citrated blood did not affect platelet deposition. Our study indicates that local thrombin generation at the site of severe injury will induce platelet activation and deposition even in the presence of average therapeutic heparin levels that inhibit blood coagulation.

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hirudin to values significantly lower than those reached by heparinized blood. Thrombin effects are localized to the thrombus-damaged wall interphase and blood does not coagulate after perfusion. The effect of hirudin on platelet thrombus formation is due to its antithrombin effect because we have excluded a direct effect of hirudin on platelets. In conclusion, our study indicates that platelet interaction to severely damaged vessel wall is heavily dependent on local thrombin activation of the platelets.

MATERIALS AND METHODS

Experimental Conditions

The animal model for the study was the Yorkshire albino pig obtained from a single local farm (body weight, 36.7 ± 1.5 kg). Twenty normal pigs were used as blood donors. They were housed in facilities of the Center for Laboratory Animal Research of our institution for 1 week before any experiment to eliminate the stress effects of transportation and change in habitat. They were individually caged in a light, temperature (20 ± 2°C) and humidity controlled environment with controlled feeding (normal pig chow; Purina Lab, St Louis, MO) and free access to water. All procedures performed in this study were approved by the appropriate institutional guidelines and followed the American Heart Association Guidelines for animal research.

After overnight fasting, blood was withdrawn and platelets were labeled with \(^{111}\)Indium-tropolone (300 to 500 μCi) as we have previously described. An average of (2 ± 0.2) \times 10^7/mL labeled platelets were injected in a final volume of 4.6 ± 0.2 mL of autologous plasma. Efficiency of the labeling procedure (in plasma) was 46% ± 3%. The labeling procedure required approximately 2 hours. Eighteen to 24 hours later, the pigs were sedated with a mixture of Ketamine and Xylazine (Ketalar, Rompun; Harney Mosby Corp, Shawnee, KS) administered intramuscularly, then intubated and ventilated (Harvard respirator), and anesthetized intravenously with sodium pentobarbital (Fort Dodge Lab, Fort Dodge, IA) with the minimal effective dose as previously described.

Through a neck incision the carotid artery was isolated and catheterized. Blood was collected for baseline determination of hematocrit (Hct) (27% ± 1%), platelet number (PN) (3.71 ± 0.24) \times 10^9/μL, mean platelet volume (MPV) (7.1 ± 0.3 μ), prothrombin time (15 ± 2 seconds), activated partial thromboplastin time (APTT) (18 ± 2 seconds), and fibrinogen (292 ± 18 mg/dL). Collected blood was also placed into the selected coagulation inhibitors. In the experiments performed with heparinized blood, the animals were intravenously heparinized (heparin so-dium injection, USP, derived from porcine intestinal mucosa; Elkins-Sinn, Inc, Cherry Hill, NJ) (120 U/kg bolus plus continuous infusion of 100 U/Kg/h for the duration of the study) and arterial blood was collected. Prothrombin time (PT) ratio in heparinized blood was 1.5 ± 0.3 and heparin levels were measured by a chromogenic substrate assay kit (KabiVitrum, Stockholm, Sweden) (1.8 ± 0.2 U/mL). Average plasma \(^{111}\)Indium activity was 2.8% ± 0.4%. Postmortem \(^{111}\)Indium-biodistribution was 48% ± 2% in blood, 26% ± 2% in liver, 12% ± 1% in spleen, 12% ± 1% in lungs, 1.7% ± 0.1% in kidneys, and 0.2% ± 0.02% in heart tissue. Serum levels of creatinine (1.3 ± 0.05 mg/dL), calcium (9.8 ± 0.1 mg/dL), total bilirubin (0.1 ± 0.01 mg/dL), cholesterol (72 ± 4 mg/dL), protein (5.2 ± 0.06 g/dL), albumin (3 ± 0.05 g/dL), glucose (131 ± 14 mg/dL), urea-nitrogen (12 ± 0.8 mg/dL), chloride (99.7 ± 1.1 meq/L), total CO2 (30 ± 0.6 meq/L), potassium (3.7 ± 1.4 meq/L), sodium (136 ± 1.4 meq/L), and triglycerides (30 ± 5 mg/dL) were measured by routine analytical chemistry assays.

To determine the PN and platelet size distribution, a Coulter P,60 platelet analyzer (Coulter, Hialeah, FL) was used. EDTA, hirudinized, heparinized acid citrate dextrose (ACD), and citrated blood from each animal were diluted 1:1000 in an isotonic buffered diluent (Thrombopet; Baker Instruments, Allentown, PA) after collection and counted during the first hour after withdrawal. After running the samples, a particle size histogram was created involving platelets and the smallest red blood cells (RBCs). The machine has preset lower (3 μm') and upper (30 μm') volume thresholds to analyze human platelets. Pig platelets and RBCs are smaller than those of human; therefore, new thresholds had to be defined. After a preliminary study, a lower and upper threshold of 1.7 μm' and 15.7 μm', respectively, were determined to include the smallest platelets and to avoid the counting of the micro-RBCs as large platelets.

Perfusion Chamber and Substrates

We have used our previously described perfusion chamber that mimics the cylindrical shape of the blood vessels. We have used our previously described perfusion chamber that mimics the cylindrical shape of the blood vessels. In the chamber, the substrate is placed in a lateral position, forming part of the blood channel by which the test surface is directly exposed to the blood. Two chambers of different internal diameters (1.0 and 2.0 mm) produce a broad range of wall shear rates on the substrate with moderate changes in average blood flow rate. In the present experiments, blood was perfused at local shear rates 212/s and 1,680/s. These shear rates range from those of patent large arteries to those of areas of moderate narrowing.

We have studied platelet interaction to superficially damaged normal pig thoracic aorta. The aorta of a deeply anesthetized pig was exposed and all branches ligated. The animal was euthanized by an overdose of anesthetic and simultaneously the aorta was perfused by cannulation of the aortic arch and the abdominal trifurcation with phosphate-buffered saline (PBS; KHPO₄, 0.1 mol/L, KH₂PO₄, 0.15 mol/L, gelatin 0.1%, pH = 7.4) containing papaverine (120 mg/L). The vessel was immediately removed and frozen in liquid N₂. Aortas were stored at −70°C and thawed in 4°C PBS the day of the experiment. This procedure induced spontaneous vessel deendothelialization. Before the experiments, segments 30 × 10 mm were separated to be placed in the chamber. Platelet deposition to severely damaged vessel wall was studied using vessel wall tunica media obtained from aortas harvested from normal untreated pigs. The aortas were processed as before, cleaned of the surrounding connective tissue, immediately deep frozen in liquid N₂, and stored at −70°C. Before the experiments, the aortas were opened longitudinally and, starting from one corner, the intima with a thin portion of the subadjacent media was lifted up and peeled off. The major portion of the exposed media that remained overlying the adventitia was then divided into 30 × 10 mm segments to be placed in the chamber as previously described for de-endothelialized vessel wall.

Collagen type I was obtained from pig Achilles tendon by a modification of the method described by Houdijk et al. for human placenta. Pig Achilles tendons were finely minced and delipidated overnight with chloroform-methanol (2:1, vol/vol). The defatted tissue was resuspended in 0.5 mol/L acetic acid (approximately 1 g in 100 mL) and digested with Pepsin (1 mg/mL) (Sigma, St Louis, MO) at 4°C for 24 hours. The pepsin-digested material was centrifuged at 30,000g for 1 hour at 4°C and redigested overnight as previously described. All clear supernatants were pooled, lyophilized, and stored. Aliquots of the lyophilized material were dissolved in 0.1 mol/L acetic acid (1 to 2 mg/mL) as needed. The collagen was precipitated by the addition of solid NaCl up to 0.9 mol/L final concentration as described by Chung and Miller.
precipitated collagens were pelleted by ultracentrifugation (30,000g; 1 hour at 4°C), pooled, and dissolved at an approximate concentration of 0.5 mg/mL in 0.1 mol/L acetic acid and stirred at 4°C for 16 hours. Collagen type I was separated by fractional salt precipitation of 0.5 mg/mL in 0.1 mol/L Tris HCl, pH = 7.5, at 4°C for about 24 hours with 8 to 10 changes of the dialysis buffer. Regularly the precipitated collagen (collagen type III) is pelleted by ultracentrifugation (20,000g, 1 hour, 4°C); however, we did not get any pellet because our starting material was Achilles tendon that is mainly constituted by collagen type I. The supernatants were pooled and precipitated by extensive dialysis against 1.5 mol/L NaCMC, mmol/L Tris HCI, and dialyzed against 20 mmol/L NaHPO₄, pH 7.5, at 4°C for about 24 hours with 8 to 10 changes of the dialysis buffer during 24 hours. The precipitated collagen was pelleted by ultracentrifugation (20,000g, 1 hour, 4°C). The pellets were dissolved in 0.1 mol/L acetic acid. Assessment of purity was performed by 5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of reduced and nonreduced samples as described by Miller and Rhodes and Butkowski et al (Fig 1). The solution containing the non-fibrillar collagens (1 mg/mL in 0.1 mol/L acetic acid) was dialyzed against 20 mmol/L NaHPO₄, pH = 7.5, at 4°C for 48 hours with 8 to 10 changes of the dialysis buffer. Fibril formation is initiated and propagated under these conditions and its completion was checked by spectrophotometry at 313 nm. Biologic activity of the fibrillar collagens was tested by whole blood platelet aggregometry by our previously reported technique. Collagen concentration was assessed by measurement of hydroxyproline, assuming that the average collagen molecule contained 10% of hydroxyproline residues.

Collagen was sprayed (Badger Air-Brush, Co, Franklin Park, IL) on plastic slides (Thomas Scientific, Swedesboro, NJ) that were divided into 30 × 10 mm segments to fit in the perfusion chamber and kept for 16 to 20 hours at room temperature. Fifty micrograms of collagen coated each plastic slide in a uniform coverage easily evidenced by stain with Commassie blue stain (protein). Staining of the collagen-coated slides after blood perfusion showed that the collagen matrix had not been dislodged or peeled off by the flow.

Experimental Procedure

Native (non-anticoagulated) blood was collected through the catheter into hirudin (20 U/mL final concentration) (Sigma), recombinant desulfatohirudin (100 and 200 U/mL) from yeast (CGP 39393) with a specific activity of 1496 ATU/mg (Ciba-Geigy, Summit, NJ), sodium citrate (9 mmol/L final concentration), and ACD (citric acid 5.4 mmol/L, sodium citrate 12 mmol/L, dextrose 9 mmol/L, final concentrations). Blood was distributed into aliquots (30 cc) and kept at room temperature until perfusion. Heparinized blood (1.8 ± 0.2 U/mL) was collected from the systemically heparinized animal and processed as described before. Blood was prewarmed in a water bath at 37°C for at least 5 minutes and then recirculated by a peristaltic pump at the preselected shear rate and a perfusion time of 5 minutes. The substrates were mounted in the chamber that was prefilled with Vassar-saline (NaHPO₄ [anhydrous] 0.2 mol/L, NaHPO₄·H₂O 0.2 mol/L, NaCl 0.1 mol/L, pH = 7.4; freshly prepared before using and filtered through Millipore [0.47 mm]) and kept at 37°C in a water bath and perfused with Vassar-saline solution for 60 seconds at 37°C. After the preperfusion period blood passed through the chamber, and at the termination of the perfusion period buffer was again passed for 30 seconds under identical flow conditions to clear away unattached cells and blood. The changes from buffer to blood and vice versa were achieved manually by a three-way valve without the introduction of stasis in the chamber. The perfused segments were fixed in a mixture of 3% glutaraldehyde in 0.2 mol/L cacodylate buffer, pH = 7.4, and counted in a gamma-well counter for quantitation of deposited platelets.

Quantitation of Platelet Deposition (PD)

Approximately 24 hours before the perfusion experiment, autologous platelets labeled with 11In (tropolone) were injected into the animal. Platelets deposited on the perfused substrate were determined by gamma-well counting of the perfused matrix and normalized by blood activity (counts), platelets counts in blood, and surface area.

Whole Blood Platelet Aggregation

Platelet aggregation was measured in whole blood (impedance) as previously reported. Briefly, blood samples were collected into a 10-cc syringe after discarding the first few milliliters of blood, and mixed with a trisodium citrate solution (3.8%, 1/10). Hct was determined in EDTA and citrated blood by the micro-Hct technique and adjusted in the citrated sample to 20% by diluting with 0.9% NaCl. All samples were kept at room temperature until tested for a period of time ranging from 15 minutes to 2 hours. One-milliliter aliquots of diluted blood were placed in a whole blood aggregometer (Chrono-Log model 540; ChronoLog, Haverton, PA) and maintained at 37°C for 3 minutes. Agonists were added at the desired concentration and the change in electrical impedance was registered on a strip chart recorder (ChronoLog). After addition of preselected doses of ADP (1 to 10 μmol/L, final concentration; ChronoLog) and collagen (1 to 5 μg/mL, final concentration; ChronoLog), the extent of aggregation was determined from the maximum height of response in ohms (10 chart paper units = 5 Ω), and the rate of aggregation from the slope at the steepest part of the curve (12/min).

Platelet Aggregation in Platelet-Rich Plasma (PRP)

Optical platelet aggregation was measured in PRP as previously described. Citrated blood was centrifuged (200g for 10 minutes) to obtain PRP, and the platelet number was adjusted to 275,000/mm³ with platelet-poor plasma (PPP). The PRP was kept at room temperature until tested for a period of time ranging from 30 minutes to 2 hours. Samples were maintained at 37°C for 3 minutes before testing. ADP (3, 5, and 10 μmol/L) and collagen (1 to 3 μg/mL) were used as agonists and aggregation was registered on a
strip chart recorder (Chronolog). Extent of aggregation was calculated from the maximum change in light transmission (%).

Ultrastructural Analysis
The specimens were fixed in a mixture of 3% glutaraldehyde in 0.2 M cacodylate buffer (pH 7.4) immediately after removal from the perfusion chamber. They were counted (γ-radiation) and processed for transmission electron microscopy. The vessels were fixed in 1% OsO4, stained in block with 2% aqueous uranylacetate, dehydrated with ethanol, and embedded in Epon 812. The tissues were examined under a Philips 201 transmission electron microscope, and representative sections were photographed with Kodak electron micrograph film 4463 (Eastman Kodak, Rochester, NY).

Data Analysis
Results are expressed as mean ± SEM unless otherwise stated. Statistical analysis was performed by Student's t-test for paired or unpaired observations when groups had equal variances (F-test) and by Mann-Whitney's U-test for groups with unequal variances (F-test). Multiple group means were compared by one or multiple factor analysis of variance with factorial or repeated measures analysis as required and differences between groups analyzed by Fisher PLSD and Scheffe-F-test.

RESULTS
Effect of Hirudin, Recombinant Hirudin r-Hirudin and Heparin on PD
The effect of selective thrombin inhibition by natural and recombinant hirudins on platelet thrombus formation was measured at low and high local shear rate conditions and compared with the platelet deposition obtained with heparinized blood.

Low Local Shear Rate
Mildly damaged vessel wall. PD on mildly damaged vessel wall in hirudin-treated blood did not differ from platelet deposition in heparinized blood (Fig 2).
Isolated collagen type I. PD on isolated collagen type I fibrils was also similar in hirudin-treated and heparinized blood (Fig 2).

Severely damaged vessel wall. PD on severely damaged vessel wall was significantly reduced by both types of hirudin (Fig 2) in comparison with heparin. The same inhibitory activity was exerted by 100 U/cc of r-hirudin than 20 U/cc of natural hirudin.

High Local Shear Conditions

Mildly damaged vessel wall. When the effects of selective thrombin inhibition by hirudin were tested at high local shear rate conditions (1,690/s), PD was not reduced by hirudin when the substrate was mildly damaged wall (Fig 3).

Isolated collagen type I. Isolated collagen type I-coated slides were perfused with blood treated with hirudin (20 U/mL), r-hirudin (100 U/mL), and heparin. PD results were similar with the three treatments (Fig 3).

Severely damaged vessel wall. PD was significantly reduced by both hirudins \( P < .001 \) compared with heparinized blood (Fig 3).

Effect of Hirudin on PD in Heparinized Blood

Blood from the same donors was heparinized, treated with r-hirudin, and treated with both heparin and r-hirudin. Bloods were perfused on the three substrates at high local shear rate conditions (Fig 4). Hirudin treatment significantly reduced PD in heparinized blood to a level obtained in blood collected in hirudin alone. Such effect was not found on mildly damaged vessel wall or isolated digested collagen, where the three treatments were similar.

Effect of the Substrate Triggering Thrombosis

There was a significant difference (two-factor ANOVA) in PD depending on the substrate triggering thrombosis.

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![Figure 3](image-url)

**Fig 3.** Mean values of PD ± SE on the three substrates at high local wall shear rate (1,690/s). Substrates were exposed to blood with different treatments/anticoagulants. Data analysis was by ANOVA. (A) Substrate, mildly damaged vessel wall (*\( P < .05 \) compared with heparin); (B) substrate, isolated collagen type I-coated slides (*\( P < .05 \) compared with heparin and hirudin; **\( P < .05 \) compared with all others); and (C) substrate, severely damaged vessel wall (*\( P < .06 \) compared with heparin; **\( P < .05 \) compared with all others). Note that different ordinate scales are being used in the figure.
(factor A, \( P < .001 \)) and the local shear rates (factor B, \( P < .001 \)) in all of the blood treatments.

At low blood shear rate (212/s), the levels of PD were of the order of 5 to 10 \( \times 10^6 \) platelets/cm\(^2\) in hirudinized and heparinized blood (Fig 2). Despite the generally low deposition values, there were differences in PD on the three substrates with the highest occurring on severely damaged vessel wall (Fig 2).

At high blood shear rate (1,690/s), PD was greater than 30 \( \times 10^6 \) platelets/cm\(^2\) on severely damaged wall (Fig 3). PD on mildly damaged vessel wall and collagen was similar (5 to 10 \( \times 10^6 \) platelets/cm\(^2\)) to that obtained at low shear rate (Fig 3).

Effect of Thrombin Inhibition by Calcium Chelation

For comparative purposes we analyzed platelet interaction to severely and mildly damaged vessel wall, and to isolated collagen type I in blood collected in in vitro used anticoagulants that exert their effect by chelating blood calcium ions.

At low shear rate, citrated blood showed similar results to heparin and hirudin with respect to platelet deposition on mildly damaged vessel wall and isolated collagen type I coated slides (Fig 2). On both substrates, blood collected in ACD showed significantly lower PD (\( P < .05 \), ANOVA and Fisher PLSD test). PD on severely damaged vessel wall was significantly reduced in citrated and ACD-blood with respect to heparinized blood (ANOVA, Fisher PLSD), while results with citrated blood were similar to those with hirudin-treated blood (Fig 3). Therefore, thrombin-mediated PD is a major mechanism of thrombus formation at the site of severe vascular injury, and it can not be prevented by systemic heparin. At high shear rate, citrated blood showed significantly lower PD than heparinized blood (ANOVA, Fisher PLSD) but similar to hirudin-treated blood, when perfusing mildly injured vessel wall and isolated collagen type I-coated slides (Fig 3). On severely damaged vessel wall, citrated blood showed significantly lower PD levels than heparinized blood and similar deposition to hirudin-treated blood. ACD-blood showed the lowest levels of PD, which were significantly lower than either heparinized or hirudinized blood (Fig 3).

Effect of Hirudin on PD in Citrated Blood

To exclude a direct effect of hirudin on platelets, blood collected in citrate (9 mmol/L final concentration) was spiked with r-hirudin (100 U/cc) and perfused on mildly and severely damaged vessel wall and isolated collagen type I-coated slides for 5 minutes at a high shear rate (1,690/s).

<table>
<thead>
<tr>
<th>Log PD (( \times 10^6/cm^2 ))</th>
<th>PN (( \times 10^3/\mu L ))</th>
<th>Hct (%)</th>
<th>MPV (( \mu ^3 ))</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A) Saline 1.6 ± 0.1</td>
<td>302 ± 17</td>
<td>24 ± 1</td>
<td>7.2 ± 0.2</td>
<td>10</td>
</tr>
<tr>
<td>Hirudin 1.4 ± 0.1</td>
<td>289 ± 12</td>
<td>25 ± 1</td>
<td>7.4 ± 0.2</td>
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</tr>
<tr>
<td>(B) Saline 1.0 ± 0.15</td>
<td>349 ± 46</td>
<td>27 ± 1</td>
<td>7.5 ± 0.4</td>
<td>4</td>
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<tr>
<td>Hirudin 1.1 ± 0.02</td>
<td>390 ± 36</td>
<td>25 ± 1</td>
<td>7.7 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>(C) Saline 0.8 ± 0.1</td>
<td>316 ± 37</td>
<td>26 ± 1</td>
<td>7.6 ± 0.3</td>
<td>4</td>
</tr>
<tr>
<td>Hirudin 0.7 ± 0.09</td>
<td>311 ± 27</td>
<td>26 ± 1</td>
<td>7.7 ± 0.3</td>
<td></td>
</tr>
</tbody>
</table>

Either saline or r-hirudin (100 U/mL) were added to citrated blood (9 mmol/L). Substrates are: (A) severely damaged vessel wall; (B) mildly damaged vessel wall; and (C) isolated collagen type I. Results are expressed as mean ± SE.

Abbreviation: N, number of experiments.
Fig 5. Rate and extent (a) of citrated whole blood platelet aggregation in control (□) and r-hirudin (100 U/mL) spiked bloods (●). There was no effect of r-hirudin in whole blood platelet aggregation. Data analysis by two factor ANOVA (A, treatment; B, agonist concentration) showed no significant effects of treatment.

We found that there were no differences in the results obtained with both blood treatments on the three biologic substrates. Therefore, hirudin did not exert any additional effect on blood platelets when thrombin generation was blocked by calcium chelation (Table 1).

Effect of Hirudin on Platelet Aggregation in Citrated Blood

The effect of hirudin on platelet function was also tested in in vitro whole blood platelet aggregation. Citrated blood with and without r-hirudin (100 U/mL) was challenged with the platelet agonists ADP (1 to 10 μmol/L) and collagen (1 to 5 μg/mL). Figure 5 shows the results obtained when collagen was used as agonist. There was no difference in maximal aggregation or rate of aggregation due to hirudin in neither collagen nor ADP-challenged platelets. Therefore, hirudin does not affect platelet function by a thrombin-independent pathway.

Platelet aggregation in isolated PRP was not affected by r-hirudin. ADP (3 to 10 μmol/L) and collagen (1 and 3 μg/mL) induced a similar extent of aggregation in control or r-hirudin–treated platelets (Fig 6).

Blood Values

There were no significant effects of either Hct or PN on the values of PD obtained (Table 2). PV, platelet mode and platelet median size distribution, and the macro–platelet region were measured in each perfusion group and the values did not show any significant difference (Table 3).

Ultrastructural Analysis

Transmission electron microscopy of vascular aortic wall, subendothelial and medial layers, was studied after perfusion with heparinized blood. Specimen perfused with non-anticoagulated blood were photographed in parallel for comparative purposes. As shown in Figs 7 through 9, fibrin is formed and deposited on severely damaged vessel wall when perfused with heparinized blood. However, fibrin formation is not seen when blood from the same donor perfused subendothelium.

DISCUSSION

This study shows that thrombin has an important role in thrombosis induced by severe (deep) arterial injury. Thrombin does not appear to mediate PD in the presence of mild arterial injury (endothelial denudation) or onto porcine type I–collagen-coated slides where PD is only a monolayer or less (< 10 x 10⁶/cm²). This study shows that the characteristics of an evolving thrombus and its inhibition will heavily depend on the underlying vascular damage. We have previously shown that flow mechanisms for deposition of platelets on various surfaces (subendothelium, Goretex, and collagen type I bundles) are substantially different and surface-dependent. The perfusion chamber used offers the advantage that different natural and synthetic surfaces and vessel wall can be simultaneously characterized as to their PD from the same donor blood at equivalent blood flow and wall shear rate. In addition, our experimental system only involves flow and substrate activation as in the vivo situation without addition of exogenous platelet-stimulating agents.

Table 2. Effect of Hct and Blood PN on PD

<table>
<thead>
<tr>
<th></th>
<th>Correlation</th>
<th>Regression</th>
<th>Determination</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DF</td>
<td>Coefficient</td>
<td>Intercept</td>
<td>Coefficient</td>
</tr>
<tr>
<td>Hct</td>
<td>451</td>
<td>0.096</td>
<td>1.6</td>
<td>0.6</td>
</tr>
<tr>
<td>Platelets (x10⁶/µL)</td>
<td>451</td>
<td>0.083</td>
<td>26.6</td>
<td>-0.3</td>
</tr>
</tbody>
</table>

Abbreviations: DF, degrees of freedom; NS, not significant.
The study of PD triggered by vessel wall or immobilized vessel wall components, under blood flow conditions that mimic conditions encountered in the arterial circulation, is critical for delineating mechanisms of thrombus formation. We analyzed the effects of thrombin inhibition by heparin, hirudin, and calcium chelation on platelet interaction to various biologic substrates at two blood local shear rate conditions in laminar-parallel streamlined flow. Heparin is an anticoagulant widely used in in vivo studies and clinical

<table>
<thead>
<tr>
<th>Table 3. Average Blood Values in the Perfusion Bloods</th>
</tr>
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<tbody>
<tr>
<td>Hct (%)</td>
</tr>
<tr>
<td>Platelet (×10^3/μL)</td>
</tr>
<tr>
<td>Platelet mode (μ^2)</td>
</tr>
<tr>
<td>Platelet median (μ^2)</td>
</tr>
<tr>
<td>MPV (μ^2)</td>
</tr>
<tr>
<td>Macro-platelet region (%)</td>
</tr>
</tbody>
</table>

Fig 7. Transmission electron microscopy (×4,500) of mildly damaged vessel wall with exposure of subendothelial structures. Heparinized blood perfused the substrate at high shear rate. Micrographs of two areas with different number of deposited platelets. Fibrin is not evident in either case.
therapy. However, heparin is a molecule that requires a cofactor (antithrombin III) to exert its antithrombin activity and has an additional variety of actions. Hirudin, a compound known for over two decades, is a specific thrombin inhibitor. Its sequence has been characterized, cloned, and expressed. This technologic advance has made available a substance difficult to obtain from its natural sources (salivary glands of leeches). Calcium chelation has been repeatedly used in in vitro blood testing to collect blood and inhibit thrombin-induced coagulation. Calcium has a variety of effects in the activation of blood factors, receptor binding, and cell activation.

Severely damaged vessel wall is a highly thrombogenic surface. PD on this substrate was the greatest regardless of the blood treatment. It was 10 orders of magnitude higher than on subendothelium or collagen-coated matrices. The exposure of tunica media to flowing blood uncovers native collagen (III and I), proteoglycans, glycosaminoglycans, elastin, and tissue-factor–producing cells in an uneven boundary layer. Those materials are exposed, although...
probably in different relative proportions, in atherosclerotic plaque rupture. In addition, the geometric configuration of the atherosclerotic vessel contributes to an increased cell activation due to the stenotic narrowing of the vessel and generation of local high shear rate. In those conditions, the activation of platelets by thrombin may have a pivotal role in thrombus formation. We found that in heparinized blood hirudin was able to reduce PD. Therefore, despite the presence of heparin there was enough generation of thrombin to induce platelet activation and deposition, but not blood clotting (Fig 4). When Ca\(^{2+}\) chelation was used as anticoagulant, the addition of r-hirudin did not reduce PD (Table 1). Thus, heparin levels able to prevent blood coagulation were unable to prevent local thrombin generation at the site of deep vessel damage when blood perfused the area at both high and low shear conditions. Because it has been reported in in vitro static assays that heparin-neutralizing material released from thromboplastin-activated platelets quenches the heparin effects,\(^{37}\) it is possible that the high levels of PF\(_4\) (heparin-neutralizing activity) at the boundaries of evolving thrombus inactivate the available heparin, thereby allowing thrombin-mediated deposition to take place.

In summary, PD on mildly damaged vessel wall and isolated collagen type I-coated matrices results in discrete cell deposition (<10 million platelets/cm\(^2\)) that is not affected by heparin, specific thrombin inhibition with hirudin, or r-hirudin and citrate. PD on severely damaged vessel wall results in macroscopic mural thrombosis (<10 million platelets/cm\(^2\)), especially at high shear rate, and is significantly reduced by hirudin (or r-hirudin) and citrate compared with heparin. Thrombus growth but not the adhesive event of platelet vessel wall interaction appears to be dependent on local thrombin production because hirudin, a specific thrombin inhibitor, seems to be more effective than heparin in the prevention of platelet-platelet interaction and thrombus growth.

To exclude a direct inhibitory effect of r-hirudin on platelets we performed experiments with calcium-chelated blood. Whole blood platelet aggregation measured by impedance or PRP platelet aggregation was not affected by r-hirudin. Similarly, r-hirudin did not affect PD in flowing blood when citrated blood was perfused on the three biologic substrates at high shear rate.

Interestingly, citrated blood gave similar results to heparinized and hirudin-treated blood in PD to mildly damaged vessel wall (exposure of subendothelial structure) and isolated collagen type I. Therefore, our results indicate that both substrates induce a mild platelet response that is not mediated by thrombin. Citrated blood gave similar results to hirudin-treated blood (lower than heparinized blood) when perfusing vessel wall with severe injury (exposure of tunica media structures). In this substrate, thrombin generation seems to be the mediator for the severe platelet activation and deposition.

ACD-anticoagulated blood appears to induce impairment of platelet function because it, unlike citrate or hirudin, reduces platelet interaction to mildly damaged vessel wall and digested collagen type I-coated slides. ACD is an in vitro used anticoagulant with acidic pH and higher citrate concentration than sodium citrate. Therefore, it lowers the plasma pH and may induce greater Ca\(^{2+}\) depletion and significantly affect platelet-receptor functions that are calcium dependent, such as the GP IIb/IIIa receptor coupling. We had previously shown a similar effect of EDTA-blood when perfusing subendothelium.\(^{18}\)
The experimental design of this protocol, comparative analysis of various substrates in an in vitro perfusion system, has precluded the study of native (non-anticoagulated) blood. However, deposition results in heparinized blood were quite comparable with those observed in native blood when perfusing mildly damaged vessel wall. Similarly, in experimental angioplasty studies, PD in areas of deep injury was not significantly reduced by heparin (50 U/kg + 50 U/kg/h) with respect to the non-anticoagulated placebo, although average results in native blood were 39% higher than in heparinized blood. In any case, heparin did not stimulate platelets or induced an increase in PD. We have previously shown that heparin at high doses is able to induce inhibition of PD to damaged vessel wall postangioplasty, and to collagen type I bundles when perfused at high shear rate for 5 minutes; but large doses of heparin are not indicated in clinical situations because of bleeding risks. These results suggest that heparin in medium-high therapeutically dosages that prevent blood coagulation and prolong APTT levels (average of 1.8 U/mL in this study) is not able to inhibit local thrombin generation at the site of severe injury because the severely injured wall appears to locally induce in situ generation of thrombin that stimulates platelets to form large thrombi. Because severe but not mild wall injury induces clinically relevant arterial platelet-related thrombosis, thrombin inhibition is likely to be a key step in its prevention.

ACKNOWLEDGMENT

We thank CIBA-Geigy and Plantorgan for their supply of recombinant hirudin. The authors thank the collaboration of Dorit Ben Moga and Carmen Busnadioge for her assistance in preparing the manuscript. We also thank Dr R. Gordon (Pathology, Mount Sinai Medical Center) for his collaboration in the electron microscopy analysis.

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Thrombin regulation of platelet interaction with damaged vessel wall and isolated collagen type I at arterial flow conditions in a porcine model: effects of hirudins, heparin, and calcium chelation

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