Hirudin Interruption of Heparin-Resistant Arterial Thrombus Formation in Baboons

By Andrew B. Kelly, Ulla M. Marzec, William Krupski, Arie Bass, Yves Cadroy, Stephen R. Hanson, and Laurence A. Harker

To determine the role of thrombin in high blood flow, platelet-dependent thrombotic and hemostatic processes we measured the relative antithrombotic and anhe mostatic effects in baboons of hirudin, a highly potent and specific antithrombin, and compared the effects of heparin, an antithrombin III-dependent inhibitor of thrombin. Thrombus formation was determined in vivo using three relevant models (homologous endarterectomy aorta, collagen-coated tubing, and Dacron vascular graft) by measuring: (1) platelet deposition, using gamma camera imaging of 111In-platelets; (2) fibrin deposition, as assessed by the incorporation of circulating 186L-fibrinogen; and (3) occlusion. The continuous intravenous infusion of 1, 5, and 20 nmol/kg per minute of recombinant hirudin (desulfatohirudin) maintained constant plasma levels of 0.16 ± 0.03, 0.79 ± 0.44, and 3.3 ± 0.77 μmol/mL, respectively. Hirudin interrupted platelet and fibrin deposition in a dose-dependent manner that was profound at the highest dose for all three thrombogenic surfaces and significant at the lowest dose for thrombus formation on endarterectomy aorta. Thrombotic occlusion was prevented by all doses studied. In contrast, heparin did not inhibit either platelet or fibrin deposition when administered at a dose that maximally prolonged clotting times (100 U/kg) (P > .1), and only intermediate effects were produced at 10-fold that dose (1,000 U/kg). Moreover, heparin did not prevent occlusion of the test segments. Hirudin inhibited platelet hemostatic function in concert with its antithrombotic effects (bleeding times were prolonged by the intermediate and higher doses). By comparison, intravenous heparin failed to affect the bleeding time at the 100 U/kg dose (P > .5), and only minimally prolonged the bleeding time at the 1,000 U/kg dose (P < .05). We conclude that platelet-dependent thrombotic and hemostatic processes are thrombin-mediated and that the biologic antithrombin hirudin produces a potent, dose-dependent inhibition of arterial thrombus formation that greatly exceeds the minimal antithrombotic effects produced by heparin.

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INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE IN ACCORDANCE WITH FEDERAL GUIDELINES (GUIDE FOR THE CARE AND USE OF LABORATORY ANIMALS, 1986). THE ANIMALS WEIGHED 8 TO 12 KG AND HAD BEEN OBSERVED TO BE DISEASE FREE FOR AT LEAST 6 WEEKS BEFORE USE. ALL ANIMALS HAD A CHRONIC ARTERIOVENOUS (A-V) SHUNT SURGICALLY IMPLANTED BETWEEN THE FEMORAL ARTERY AND VEIN. FOR SHUNT PLACEMENT, ATRIPINE (0.04 MG/KG INTRAMUSCULARLY) WAS ADMINISTERED PREANESTHETICALLY, AND KETAMINE (10 MG/KG INTRAMUSCULARLY) AND DIAZEPAM (0.5 MG/KG INTRAVENOUSLY) WERE USED TO INDUCE GENERAL ANESTHESIA WITH HALOTHANE IN OXYGEN.

THE PERMANENT SHUNT SYSTEM CONSISTED OF TWO 5-CM LENGTHS OF SILICONE RUBBER TUBING, 5.0 MM INTERNAL DIAMETER (ID) (SILASTIC; DOW CORNING CORP, MIDLAND, MI). BLOOD FLOW WAS ESTABLISHED BY CONNECTING THE TWO SILICONE RUBBER SHUNT SEGMENTS WITH A 2-CM LENGTH OF BLUNT-EDGE TEFLOMN TUBING (2.8 MM ID) (SMALL PARTS, INC, MIAMI, FL). AS DESCRIBED PREVIOUSLY, THE CHRONIC SHUNTS OF THIS DESIGN DO NOT DETECTABLY SHORTEN PLATELET OR FIBRINOGEN REMOVAL RATES AND DO NOT PRODUCE MEASURABLE ACTIVATION OF PLATELETS OR FIBRINOGEN.10-27 SEGMENTS OF ENDARTERECTOMIZED AORTA, COLLAGEN-COATED TUBINGS, OR DACRON VASCULAR GRAFTS WERE SUBSEQUENTLY INTERPOSED BETWEEN THE ARMS OF THE PERMANENT A-V SHUNT.

PLATELET COUNTS AND HEMATOCRIT DETERMINATIONS WERE PERFORMED ON WHOLE BLOOD COLLECTED IN 2 MG/M TO DISODIUM EDTA USING A J.T. BAKER (ALLENTOWN, PA) MODEL 810 WHOLE BLOOD ANALYZER. MEAN PLATELET COUNTS WERE 319,000 ± 92,000 PLATELETS/µL AND HEMATOCRITS AVERAGED 34 ± 5%. BLEEDING TIME MEASUREMENTS WERE PERFORMED IN DUPLICATE ON THE SHAVED VOLAR SURFACE OF THE FOREARM USING THE STANDARD TEMPLATE METHOD AS previoulsy described.16,26

ENDARTERECTOMIZED AORTIC SEGMENTS. HOMOLOGOUS BABOON AORTA (4 TO 5 MM ID) WAS FLUSHED IMMEDIATELY WITH SALINE ON REMOVAL AND DIVIDED INTO 3-CM LENGTHS. ENDARTERECTOMIZED SEGMENTS WERE PREPARED AS DESCRIBED PREVIOUSLY.26 IN BRIEF, EACH AORTIC SEGMENT WAS INVERTED OVER A PAIR OF CURVED FORCES AND PINNED TO A FIRM WORKING SURFACE. THE INTIMA TOGETHER WITH THE INTERNAL ELASTIC LUMINA WERE REMOVED OVER A CENTRAL 1-CM LENGTH, USING SHARP DISSECTION. EACH SEGMENT WAS THEN RETURNED TO ITS NORMAL CONFIGURATION. EACH END OF THE VESSEL SEGMENT WAS CANNULATED USING FLANGED TEFLOMN TUBING (HEAT SHRINKABLE TEFLOMN, SMALL PARTS, INC, MIAMI, FL). THE ENDS OF EACH VESSEL WERE SECURED TO THE TEFLOMN TUBINGS USING 3-0 SILK TIES. A 5-CM LENGTH OF SILICONE RUBBER TUBING (DOW CORNING INC, CORNING, NY) WAS PLACED IN EACH TEFLOMN CANNULA AND SECURED BY HEAT-SHRINKING THE NONFLANGED ENDS OF TEFLOMN TUBING OVER THE SILICONE RUBBER WITH A BUNSEN FLAME. THE VESSEL WAS FURTHER ENCLOSING BY PLACING TUBING (SMALL PARTS, INC) OVER THE APPARATUS. THE RESULTANT APPARATUS ALLOWED MAINTENANCE OF THE VESSEL IN A CYLINDRICAL CONFIGURATION WITH A SMOOTH TRANSITION BETWEEN THE VESSEL AND CONNECTING TUBINGS. CONTROL AORTIC SEGMENTS WERE SIMILARLY MANIPULATED WITHOUT PERFORMING THE ENDARTERECTOMY.

DACRON VASCULAR GRAFT SEGMENTS. EXTERNALLY SUPPORTED UNCRIMPED KNITTED DACRON GRAFTS (SAUVAGE EXTERNAL VELOUR, MEAN POROSITY 2,000 TO 2,200 ML/H, O/MIN AT 120 MM Hg) WERE OBTAINED FROM C.R. BARD, INC (BILLERICA, MA). ALL GRaFTS WERE 5 CM IN LENGTH, 4.0 MM ID. BEFORE EVALUATION IN THE ARTERIOVENOUS SHUNT SYSTEM, THE GRaFTS WERE RENDERED IMPERVIOUS TO BLOOD LEAKAGE BY AN EXTERNAL WRAPPING OF PARAFILM (AMERICAN CAN COMPANY, NEW YORK, NY) AND PLACED INSIDE A 5-CM LENGTH OF 5.3 MM ID. "HEAT-SHRINK" TEFLOMN TUBING.23,24

COLLAGEN-COATED TUBULAR SEGMENTS. TO PREPARE THE COLLAGEN-COATED TUBING, A SOLUTION OF COLLAGEN (18 MG/M IN PHOSPHATE-BUFFERED SALINE [PBS], A GIFT FROM COLLAGEN CORP, PALO ALTO, CA) WAS INFLICTED TO FILL 4 MM ID.-2 CM LONG SEGMENTS OF GORE-TEX VASCULAR GRAFT (30 µM INTERNOVAL POR PORE SIZE) AND INCUBATED AT 37°C FOR 30 MINUTES TO FORM INSOLUBLE COLLAGEN FIBRILLs. THE CORE OF COLLAGEN GEL WAS REMOVED BY BALLOON CATHETER, LEAVING A SMOOTH LUMINAL COATING OF COLLAGEN FIBRILLs ANCHORED IN THE PORES OF THE VASCULAR GRAFT. THE COLLAGEN-COATED GRaFTS WERE THEN IMMOBILIZED BY CROSSLINKING WITH GLUTARALDEHYDE SOLUTION (1% IN PBS AT 4°C) FOR 12 TO 18 HOURS. THE TUBING WAS WASHED EXTENSIVELY WITH STERILE SALINE BEFORE USE. BEFORE PLACEMENT IN THE EXTERIORIZED A-V SHUNT, 2-CM SEGMENTS OF COLLAGEN-COATED TUBING WERE COUPLED TO SILICONE RUBBER AS A BUTT JOINT USING HEAT-SHRINK TEFLOMN CASING. PREPARED IN THIS WAY, COLLAGEN-COATED TUBING INDUCED PLATELET DEPOSITION OVER A 60-MINUTE INTERVAL THAT WAS EQUIVALENT TO RESULTS OBTAINED WITH RAT SKIN COLLAGEN AS REPORTED PREVIOUSLY.26 (3.10 ± 1.37 X 3.07 ± 1.52, RESPECTIVELY; P > .8) AND SIMILAR TO THE ACCUMULATION OF PLATELETS ON ENDARTERECTOMIZED AORTIC SEGMENTS (SEE RESULTS).

MEASUREMENTS OF THROMBUS FORMATION. MEAN BLOOD FLOW RATES THROUGH AORTIC, COLLAGEN, AND VASCULAR GRAFT SEGMENTS INCORPORATED INTO THE A-V SHUNT SYSTEM WERE MEASURED CONTINUOUSLY USING A DOPPLER ULTRASONIC FLOWMETER (L & M ELECTRONICS, DALY CITY, CA). IN ALL STUDIES, INITIAL BLOOD FLOW RATES RANGED FROM 140 TO 230 ML/MIN. DURING EACH STUDY, OCCLUSION WAS SCORED WHEN THE MEAN BLOOD FLOW RATE DECREASED TO LESS THAN 10 ML/MIN. SEGMENTS OF ENDARTERECTOMIZED AORTA, COLLAGEN-COATED TUBING, AND DACRON VASCULAR GRAFT WERE INITIALLY PLACED AND IMAGED NO SOONER THAN 1 HOUR AFTER THE INJECTION OF AUTOLOGOUS 111INDUROTAILED PLATELETS. WHEN HIRUNID OR HEPARIN WAS GIVEN, THE THROMBOGENIC SEGMENTS WERE PLACED 15 MINUTES AFTER THE START OF INTRAVENOUS ADMINISTRATION. THE SEGMENTS WERE IMAGED UNTIL OCCLUSION, OR FOR 1 HOUR, AND THEN REMOVED.

AUTOLOGOUS BABOON PLATELETS WERE LABELED WITH 111INDIUM-OXINE ACCORDING TO A MODIFICATION OF THE METHOD OF KOTZE ET AL. BRIEFLY, 85 ML OF BLOOD WAS COLLECTED DIRECTLY INTO SYRinges CONTAINING 15 ML ACID-CITRATE-DEXTRAN ANTICOAGULANT (NATIONAL INSTITUTES OF HEALTH FORMULA A). PLATELET-RIICH PLASMA (PRP) WERE OBTAINED BY CENTRIFUGATION OF THE WHOLE BLOOD AT 180G FOR 15 MINUTES. THE RBCs WERE RESUSPENDED TO THEIR ORIGINAL VOLUME WITH RINGER’S CITRATE DEXTROSE (RCD, pH 6.5) AND WASHED THREE TIMES BY CENTRIFUGATION AT 750G FOR 3 MINUTES. THE PRP AND SALINE HArVESTS WERE POOLED AND CENTRIFUGED AT 750G FOR 25 MINUTES TO FORM A PLATELET PELLEt. AFTER THE SUPERNATANT WAS DISCARDED, THE PLATELET PELLEt WAS GENTLY RESUSPENDED IN 5 ML RCD. TO REMOVE CONTAMINATING RBCs, THE RESUSPENDED PLATELETS WERE CENTRIFUGED AT 300G FOR 4 MINUTES. THE SUPERNATANT PLATELET SUSPENSION WERE INCUBATED AT ROOM TEMPERATURE FOR 30 MINUTES WITH 1 MCI 111INDIUM-OXINE (AMESHURM CORP, ARlington HEIGHTS, IL). THE NONPLATELET-BOUND ISOTOPE WERE THEN DISCARDED AFTER A FINAL CENTRIFUGATION AT 750G FOR 25 MINUTES. THE Labeled PLATELETS WERE SUBSEQUENTLY RESUSPENDED IN 5 ML RCD AND INJECTED WITHIN 30 MINUTES. HARVESTED RBCs WERE ALSO RETURNED AT THIS TIME. THE AVERAGE LABELING EFFICIENCY BY THIS METHOD WAS GREATER THAN 90%. PREVIOUS STUDIES WITH THIS METHOD HAVE SHOWN THAT THE LABELLED PLATELET POPULATION IS FUNCTIONALLY NORMAL, AS DEMONSTRATED BY EQUIVALENT REDUCTIONS IN CIRCULATING PLATELET COUNT AND CIRCULATING PLATELET RADIOACTIVITY IN RESPONSE TO INFUSED COLLAGEN SUSPENSIONS OR BLOOD EXPOSURE TO VASCULAR GRaFTS.30,27 IMAGES OF THE ENDARTERECTOMIZED AORTIC SEGMENTS, COLLAGEN-COATED TUBINGS, AND DACRON VASCULAR GRAFTS, INCLUDING PROXIMAL AND DISTAL SILICONE RUBBER SEGMENTS, WERE ACQUIRED WITH A PICKER DC 4/11 DYNA SCINTILLATION CAMERA (PICKER CORP, NORTHFIRD, CT), AND STORED AND ANALYZED ON A MEDICAL DATA SYSTEMS A1 Computer (MEDTRONIC, ANN ARBOR, MI), WHICH INTERFACED WITH THE CAMERA. IMAGES WERE ALSO ACQUIRED OF COMPAREABLY CONFIGURED DEVICES FILLED WITH AUTOLOGOUS BLOOD OF KNOWN VOLUME (BLOOD STANDARD). THE ACTIVITIES OF THE STANDARD AND THE THROMBOGENIC SEGMENTS WERE COUNTED IN THE SAME REGION OF INTEREST, AS DEFINED BY IMAGE ANALYSIS SOFTWARE ROUTINES.30,26,27 IMAGEx WERE ACQUIRED AT 5-MINUTE INTERVALS. DOPWSTED 111-INPLATELET ACTIVITY, CALCULATED BY SUBTRACTING THE BLOOD STANDARD ACTIVITY FROM ALL DYNAMIC STUDY IMAGES, INCREASED
monotonically over the exposure period. The total number of platelets deposited after 1 hour (labeled plus unlabeled cells) was calculated by dividing the deposited $^{111}$In-platelet activity by the blood standard platelet activity and multiplying by the volume of the blood standard and the circulating platelet count (platelets per milliliter). The results were expressed as platelets deposited per centimeter.

The concentration of fibrinogen was estimated spectrophotometrically by a modification of Jacobson's method, in which the optical density of thrombin-coagulable protein was determined after collection on a glass rod and subsequent solution in alkaline urea.

To quantify the formation of fibrin on the collagen and vascular graft segments, we injected homologous $^{125}$I-fibrinogen (approximately 5 μCi) intravenously 15 minutes before incorporating the thrombogenic segments into the A-V shunts. Homologous fibrinogen was purified by β-alanine precipitation and labeled with $^{125}$I using the iodine monochloride method. In vivo this preparation was greater than 95% clottable and was functionally equivalent to unlabeled fibrinogen. At the time of segment exposure, blood was collected in EDTA for the determination of both $^{125}$I-fibrinogen activity and fibrinogen concentration from which specific activity was calculated. After a 1-hour exposure to flowing blood, the collagen and vascular graft segments were removed, placed in 2% glutaraldehyde fixative, and stored at 4°C. After allowing 30 minutes for the $^{111}$In-activity to decay (half-life 2.8 days), the $^{125}$I-activity in each segment was counted. Deposited fibrin (mg) was calculated by dividing by the $^{125}$I-activity in the segment (cpp) by the clottable plasma $^{125}$I-activity (cpp/ml) and multiplying by the plasma fibrinogen level (mg/ml). Results were expressed as total fibrin (mg/cm) contained in the thrombus associated with each segment.

Desulfatohirudin, a recombinant product expressed in yeast, was a gift from CIBA-Geigy Pharmaceuticals (Horsham, UK). This polypeptide is similar to natural hirudin, differing only by the absence of a sulfate group on Tyr,., Desulfatohirudin was prepared as a sterile saline solution and infused intravenously for 1 hour at doses of 1, 5, and 20 nmol/kg/min.

Heparin was administered at two different doses: (1) an initial bolus of 100 U/kg given 5 minutes before the study with subsequent infusion of 10 U/kg per hour, and (2) a bolus injection increased by 10-fold (1,000 U/kg). The lower dose of heparin was selected to prolong and maintain the activated partial thromboplastin time (APTT) at times generally observed during cardiopulmonary bypass procedures and at values exceeding those produced by the low and intermediate doses of hirudin.

To determine the plasma antithrombin activity levels of hirudin, blood was collected in 3.8% sodium citrate (9 parts to 1) and immediately centrifuged. The plasma was placed on ice and assayed immediately or flash frozen at −70°C for subsequent assay of hirudin’s antithrombin activity. Plasma samples containing unknown concentrations of hirudin antithrombin activity were related to a standard curve for hirudin, generated with autologous pretreatment plasma. Heparin levels were measured as anti-Xa activity by chromogenic assay (Stachrom Heparin, Stago, France).

Plasma levels of the platelet specific α-granule proteins PF4 and βTG, and FPA, a thrombin cleavage product of fibrinogen, were determined by radioimmunoassay on blood samples collected and processed as previously described.

All statistical analyses were performed using the PROPHET system of the Division of Research Resources, National Institutes of Health (Bethesda, MD). Statistical comparisons were made using the Student’s t-test (two-tailed) for paired and unpaired sample groups when the data were normally distributed. Remaining data were compared using the Wilcoxon’s sign rank test. All data in the Results section are given as the mean ± 1 standard error of the mean.

RESULTS

Each of five animals bearing segments of collagen-coated tubing and Dacron vascular graft were evaluated in control nontreatment studies and during the infusion of intermediate-dose (5 nmol/kg/min) and high-dose (20 nmol/kg/min) hirudin on serial days in random sequence. These doses were selected to produce intermediate and marked prolongation of the activated partial thromboplastin time (Table 1). Each of three additional animals bearing aortic segments received hirudin at doses of 1 and 5 nmol/kg/min. The blood levels of hirudin antithrombin activity remained constant throughout the 60-minute period of continuous infusion for 1, 5, or 20 nmol/kg/min (0.16 ± 0.03, 0.79 ± 0.44, and 3.3 ± 0.77 μmol/L, respectively). There were no changes in the fibrinogen level or the platelet count (Table 1) during the experiments, and no detectable effects on blood pressure, pulse rate, or EKG patterns were observed.

In each of five animals, the effects of two different doses of intravenous hirudin were studied (100 U/kg bolus followed by infusion of 10 U/kg per hour or a bolus injection of 1,000 U/kg) and compared with untreated control results. The lower dose of hirudin produced a steady-state blood level of 1.0 ± 0.06 U/mL and marked prolongation of the APTT comparable with that produced during intense clinical heparin therapy and approaching that produced by the highest dose of hirudin (Table 1). The higher dose of hirudin prolonged the APTT to greater than 300 seconds throughout the 60-minute period of study.

Bleeding times were prolonged by hirudin at the intermediate and high doses (12.1 ± 5.9 and 13.8 ± 4.1, respectively; P < .001, baseline v either treatment). The difference in bleeding times with the two doses of hirudin was not significant (P > .2). By contrast, bleeding times were not prolonged by hirudin at 100 U/kg (Table 1, P > .5), and the

<table>
<thead>
<tr>
<th>Measurements</th>
<th>Untreated Controls</th>
<th>Heparin Administered</th>
<th>Desulfatohirudin Administered</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100 U/kg</td>
<td>1,000 U/kg</td>
<td>1 nmol/kg/min</td>
</tr>
<tr>
<td>Plasma level</td>
<td>0</td>
<td>1.0 ± 0.08</td>
<td>0.16 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>(U/mL)</td>
<td>(U/mL)</td>
<td>(μmol/mL)</td>
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<tr>
<td>Platelet count</td>
<td>319 ± 92</td>
<td>327 ± 92</td>
<td>328 ± 79</td>
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<tr>
<td>Bleeding time (min)</td>
<td>4.7 ± 1.1</td>
<td>5.0 ± 1.5</td>
<td>8.5 ± 0.3</td>
</tr>
<tr>
<td>APTT (s)</td>
<td>35 ± 4</td>
<td>238 ± 38</td>
<td>&gt; 300</td>
</tr>
<tr>
<td>Plasma fibrinogen (g/L)</td>
<td>3.42 ± 0.64</td>
<td>3.63 ± 0.46</td>
<td>3.66 ± 0.55</td>
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</table>

Table 1. Comparison of Antithrombotic Effects of Recombinant Desulfatohirudin and Heparin
1,000 U/kg dose of heparin minimally prolonged the bleeding time (8.5 ± 0.3 minutes; P < .05 vs control average of 4.7 ± 1.1 minutes, and the lower dose of heparin).

Thrombus formed rapidly on segments of endarterectomized aorta, collagen-coated tubing, and Dacron vascular graft in untreated control studies, reaching comparable values and consistently producing occlusion by 60 minutes (Table 2, Fig 1).

Platelet deposition was interrupted by hirudin in a dose-dependent manner for segments of endarterectomized aorta, collagen-coated tubing, and vascular grafts; the reduction at the highest dose was striking (Fig 1, Table 2). Of interest, the lowest dose of hirudin (1 nmol/kg/min) substantially reduced platelet deposition on endarterectomized aorta (Fig 1). Hirudin also reduced fibrin deposition on both collagen-coated tubing and Dacron vascular grafts, although hirudin (5 nmol/kg/min) abolished fibrin deposition in the former case but produced only intermediate effects in the latter. Whereas occlusion was observed in all of the control studies (16 of 16), none of the shunts bearing endarterectomized aortic segments (0 of 6), collagen-coated tubings (0 of 10), or Dacron vascular grafts (0 of 10) became occluded when the animals received hirudin.

Table 2. Comparison of Antithrombotic Effects of Recombinant Desulfatohirudin and Heparin

<table>
<thead>
<tr>
<th>Measurements</th>
<th>Untreated Control</th>
<th>Heparin Administered 100 U/kg</th>
<th>Desulfatohirudin Administered 5 nmol/kg/min</th>
<th>20 nmol/kg/min</th>
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<tbody>
<tr>
<td></td>
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</tr>
<tr>
<td><strong>Plasma level</strong></td>
<td>0</td>
<td>1.0 ± 0.08 (μmol/mL)</td>
<td>0.79 ± 0.44 (μmol/mL)</td>
<td>3.3 ± 0.7</td>
</tr>
<tr>
<td><strong>Platelet deposition</strong></td>
<td>3.07 ± 1.52 (μmol/mL)</td>
<td>2.4 ± 0.20 (μmol/mL)</td>
<td>0.94 ± 0.09 (μmol/mL)</td>
<td>0.15 ± 0.1</td>
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<tr>
<td><strong>Fibrin deposition</strong></td>
<td>0.81 ± 0.40 (μg/cm)</td>
<td>0.63 ± 0.20 (μg/cm)</td>
<td>0.04 ± 0.02 (μg/cm)</td>
<td>0.01 ± 0.0</td>
</tr>
<tr>
<td><strong>PF4 (μg/L)</strong></td>
<td>4.8 ± 3.7</td>
<td>4.6 ± 2.6 (μg/L)</td>
<td>4.1 ± 1.9 (μg/L)</td>
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<tr>
<td><strong>βTG (μg/L)</strong></td>
<td>19.5 ± 5.4</td>
<td>16.1 ± 3.4 (μg/L)</td>
<td>10.6 ± 7.2 (μg/L)</td>
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<tr>
<td><strong>FPA (nmol/L)</strong></td>
<td>0.20 ± 0.04</td>
<td>0.04 ± 0.02 (nmol/L)</td>
<td>0.01 ± 0.0 (nmol/L)</td>
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*Not measured because heparin elevates plasma PF4 levels.

**DISCUSSION**

Our findings show that intravenous hirudin, given as recombinant desulfatohirudin, a potent and specific inhibi-
tor of thrombin, interrupts platelet-rich, arterial-type, high-flow thrombus formation on endarterectomized aortic segments, collagen-coated tubings, and Dacron vascular grafts in a dose-dependent manner without observable cardiovascular or other adverse consequences in baboons. The antithrombotic effects were most striking for endarterectomized aorta (Fig 1). However, platelet-plug formation was also significantly impaired by hirudin, as evidenced by prolonged bleeding times. The potent antithrombotic effects produced by this biologic antithrombin contrast with the resistance of these platelet-dependent thrombotic and hemostatic processes to the administration of heparin.

The models used in this study were designed to simulate clinically relevant arterial thrombus formation by exposing native flowing blood to: (1) acutely endarterectomized arterial (medial) wall structures; (2) collagen surfaces; and (3) prosthetic vascular graft material. Flow rates averaged 140 to 230 mL/min, producing wall shear rates ranging from 400 to 600 seconds⁻¹. These shear rates are similar to those found in large- and medium-sized arteries. Thrombus formation in each of these models is resistant to aspirin therapy or the combination of aspirin plus heparin.26 Despite the compositional differences in the three thrombogenic segments, platelet and fibrin deposition were quantitatively quite similar. Presumably, this similarity is related to the observation that after the initial deposition of thrombotic material, subsequent accumulation reflects recruitment of platelets on already-formed thrombus, a process that is common for each of the three different thrombogenic segments at that time. Interestingly, the relative antithrombotic effects of hirudin were clearly different for the various thrombogenic segments in that thrombus formation following arterial endarterectomy was more responsive to hirudin than that observed with Dacron vascular grafts, and fibrin deposition was more effectively interrupted than was platelet accumulation. The increased thrombogenicity of the Dacron vascular graft segments may reflect the generation of large amounts of thrombin within the interstices of the knitted graft material as opposed to the limited production of thrombin on the smooth-walled collagen or vascular medial cells.

The systemic blood markers of thrombus formation in vivo, PF4, βTG, and FPA, were clearly increased by thrombus formation in control studies; hirudin administration blocked the elevation of these products in circulating blood (Table 2). However, rigorous collection and processing procedures were required to minimize artifacts because the levels may be confounding if activation in vitro is variable. Thus, paired basal and 60-minute samples were obtained for reliable comparisons.

In vivo, desulfatohirudin was rapidly cleared from the circulation. Given the infusion rates required to maintain steady-state antithrombotic plasma levels and the rates of clearance after continuous intravenous infusion (data not shown), the half-life of desulfatohirudin was estimated to be 4 to 5 minutes in baboons. These findings suggest that subcutaneous administration may not be efficient or practical for achieving the blood levels necessary to interrupt platelet-dependent thrombotic processes, as opposed to the substantially smaller doses required for the inhibition of venous-type thrombus formation.28 Natural hirudin, the most potent antithrombin known in nature, is a 65-residue polypeptide that interacts specifically with thrombin to form an inactive, noncovalent enzyme-inhibitor complex with a very low dissociation constant (kd = 20 pmol/L).15-18 Desulfatohirudin, the recombinant product used in the present study, differs from the natural material only in that it lacks the sulfate group on Tyr63.16 In vitro this recombinant hirudin retains potent antithrombin activity (kd = 200 pmol/L) with many orders of magnitude greater selectivity for thrombin as opposed to other serine proteases.14-19 Despite the 10-fold decreased affinity of desulfatohirudin for thrombin, in vivo this recombinant material has been shown to exhibit antithrombotic efficacy similar to that of heparin for inhibition of venous thrombus formation and intravascular coagulation in mice, rats, pigs, and dogs when administered intravenously or subcutaneously at doses that comparably prolong the partial thromboplastin time.14,16,34,35 The present data are also in accord with the observations that hirudin blocks thrombus formation induced by angioplasty in pigs.35 The capacity of hirudin to interrupt platelet-mediated thrombotic processes is similar to the antithrombotic effects produced by the synthetic antithrombin D-phenylalanyl-L-prolyl-L-arginyl-chloromethyl ketone (D-FPRCH,C).31,36 Like hirudin, that agent also inhibits platelet-dependent, thrombus formation, and impairs platelet hemostatic function in the baboon.57 Although the inhibitory capacity of D-FPRCH,C is relatively specific for thrombin over other activated serine proteases in the coagulation cascade, some nonspecificity might be expected at the doses used in the reported study.56 Thus, the preeminent role of thrombin in mediating arterial thrombotic processes is more convincingly shown by the present experimental results using hirudin.

Desulfatohirudin may have some advantages over synthetic antithrombins as a potential antithrombotic agent. First, subcutaneous administration of hirudin maintains blood levels adequate for inhibition of venous thrombosis, thereby avoiding the necessity for intravenous infusion that might be required for other antithrombins. However, delayed removal of hirudin may be a potential problem if bleeding complications develop because no antidote is currently available. Second, fully antithrombotic doses of hirudin produce only an intermediate prolongation of the bleeding time, whereas other antithrombins may produce a marked prolongation of the bleeding time at comparable antithrombotic doses.28 Third, although toxicity data are limited, systemic hirudin may be theoretically less toxic than some antithrombins. For example, D-FPRCH,C may produce secondary mutagenic complications related to its alkylating activity. On the other hand, hirudin’s origin from a species far removed from humans suggests that its administration may induce hirudin-specific antibodies that would preclude retreatment.

Recombinant desulfatohirudin may have therapeutic
applications when potent, immediate, and transient antithrombotic therapy is needed for heparin- and aspirin-resistant arterial thrombotic processes. For example, hirudin applications when potent, immediate, and transient angioplasty, endarterectomy, endovascular stenting, small-caliber graft implantation, or reocclusion after successful thrombolytic reperfusion of acutely thrombosed arteries. Additionally, hirudin may be useful in patients with heparin-induced thrombocytopenia who require acute anticoagulant therapy for cardiopulmonary bypass or venous thromboembolic disease.

In summary, we conclude that thrombin has an important mediating role in acute thrombus formation under arterial flow conditions and that interrupting thrombin activity with desulfathohirudin may produce an antithrombotic benefit associated with a significant impairment of hemostatic function.

REFERENCES


32. Jacobsson K I: Studies on the determination of fibrinogen in...


Hirudin interruption of heparin-resistant arterial thrombus formation in baboons

AB Kelly, UM Marzec, W Krupski, A Bass, Y Cadroy, SR Hanson and LA Harker