A Four-Hour Infusion of Recombinant Hirudin Results in Long-Term Inhibition of Arterial-Type Thrombosis in Baboons

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Intravenous recombinant (r-)hirudin has a potent antithrombotic effect in aspirin- and heparin-resistant platelet-dependent thrombus formation in baboon models. However, these thrombi reform when therapy is stopped after 60 minutes. To determine if 4 hours of therapy can produce a lasting antithrombotic effect, we investigated the extent of deposition of 125I-labeled platelets onto 0.5-cm² segments of Dacron vascular grafts for 53 hours. These grafts had been incorporated into exteriorized permanent femoral arteriovenous shunts in baboons. Platelet deposition in eight untreated animals was generally sigmoidal. Maximum platelet deposition, 1.7% ± 0.9% of injected labeled platelets, was reached after approximately 4 hours. Deposition then gradually decreased to 0.4% ± 0.2% of injected labeled platelets after 53 hours. After a thrombus was allowed to form for 15 minutes in six animals, intravenous treatment with r-hirudin at a dose of 20 nmol (0.14 mg)/kg-min⁻¹ (aPTT >300 seconds) was started and maintained for 4 hours. Platelet deposition was interrupted during treatment. After infusion was stopped, platelets accumulated again, but not as much as in the untreated animals. Maximum platelet deposition, 0.7% ± 0.2% of injected labeled platelets, was significantly less (P < .01), and was reached after approximately 23 hours. Therefore, deposition decreased to 0.4% ± 0.2% at 53 hours. The shunts in all of the untreated animals occluded at some stage during the study, while only one shunt occluded in the treated animals. We conclude that 4 hours of treatment of a freshly formed thrombus with a full antithrombotic dose of r-hirudin markedly reduced the maximum thrombus size and the rate of initial platelet deposition after treatment was stopped. This approach could produce lasting benefits in certain clinical settings. © 1995 by The American Society of Hematology.

T HROMBIN PLAYS A pivotal role in thrombosis and hemostasis. It amplifies the thrombotic process by activation of platelets and coagulation factors V and VIII. It also stabilizes and enlarges the thrombotic mass by cleavage of fibrinogen and activation of factor XIII. Fibrin-bound thrombin mediates platelet recruitment into existing thrombi and promotes further activation of coagulation. When complexed to recombinant (r-)hirudin, thrombin loses its effects on platelets and coagulation, and thrombosis cannot proceed. Therefore, it is not surprising that r-hirudin, in high enough dosages, interrupts platelet-dependent arterial-type thrombosis. Although the studies clearly show that r-hirudin inhibits acute arterial thrombosis, it is not clear what the effect on thrombosis is after treatment was stopped, especially in view of its short half-life in plasma. In one study where a native Dacron vascular graft (Bard Vascular Systems Division, Billerica, MA) was treated with D-Phe-Pro-Arg chloromethyl ketone (PPACK), a specific thrombin inhibitor with a similar effect on platelet deposition than r-hirudin, platelet deposition proceeded at the same rate as in the untreated studies after treatment was stopped and reached approximately the same level of deposition after 1 hour. In another study where a 15-minute old thrombus was treated with either r-hirudin or PPACK for 1 hour, platelets deposited at a slower rate (untreated studies) after treatment was stopped and approximately 20% to 40% less platelets were deposited onto the thrombus during the following 3 hours when compared with the untreated animals.

In this study, we investigated if 4 hours of treatment with a full antithrombotic dose of r-hirudin may result in long-term inhibition of platelet deposition after treatment was stopped. This was done in a baboon model of arterial thrombus formation. The baboon was selected as the experimental model because of its similarity to humans with respect to the molecular and cellular composition and function of the hemostatic apparatus. Thrombogenic devices consisting of Dacron vascular graft material were inserted as extension segments into chronic femoral arteriovenous access shunts. Systemic treatment with r-hirudin was started 15 minutes after the devices were placed and maintained for 4 hours. The devices were kept in place for up to 53 hours. Thrombogenesis was assessed by measuring the deposition of 125I-labeled platelets onto the thrombogenic device for the first 6 hours after placement and then twice daily.

MATERIALS AND METHODS

Animals studied. Thirteen normal male baboons (Papio ursinus) were used. The animals weighed 10 to 15 kg and were disease-free for at least 6 weeks before use. All procedures were approved by the Institutional Ethics Committee for Animal Experimentation in accordance with Federal Guidelines (Guide for the Care and Use of Laboratory Animals, Public Health Service National Institutes of Health publication number 85-23, 1985). The baboons supported permanent Teflon-Silastic arteriovenous (AV) shunts (Ven Products, Johannesburg, South Africa) implanted in the femoral vessels. These shunts do not detectably shorten platelet survival or produce measurable platelet activation. In addition, we used normal baboons without AV shunts to measure the mean platelet life span.

Study protocol. A thrombus was generated using uncrimped, knitted Dacron vascular graft material (0.5 cm²) that was built into the wall of silicone rubber tubing (4 mm inside diameter). The method to construct the thrombogenic device is described in detail. In each experiment, a thrombogenic device, prefilled with saline to avoid a blood-air interface, was incorporated as an extension segment into the permanent AV shunt by means of Teflon connectors. The devices were kept in place for 53 hours in both the untreated (n = 8) and treated (n = 6) baboons. Blood flow rate through the

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device was 100 to 140 mL/min, producing wall shear rates similar to that found in medium to large sized arteries.15 In the treatment studies, r-hirudin (Hoechst AG, Frankfurt and Behringwerke AG, Marburg, Germany) was systematically infused at a rate of 20 nmol/kg-min-1 for 4 hours. Treatment was started 15 minutes after placement of the thrombogenic device. The r-hirudin was dissolved in saline and infused into the tubing of the AV shunt distal to the thrombogenic device.

**Graft imaging and quantification of platelet deposition.** Autologous platelet sheets were labeled with 111In-chloride as previously described.17 Imaging and quantification of the deposition of 111In-platelets were done as described in detail.11,18 Image acquisition of the grafts, including proximal and distal silastic segments, was done with a Searle Pho scintillation camera (Siemens Medical Division, Hoffman Estates, IL) fitted with a high-resolution collimator. The images were stored and analyzed with a Medical Data Systems A computer (Medtronic, Ann Arbor, MI) interfaced with the camera. Dynamic image acquisition, 10-minute images (128 × 128 byte mode) for 6 hours, was started simultaneously with the start of blood flow through the devices. Thereafter, a static image of 10 minutes (128 × 128 byte mode) was acquired twice daily until the end of the study. A 10-minute image (128 × 128 byte mode) of a 3 mL autologous blood sample to determine circulating blood radioactivity (blood standard) was also acquired each time the grafts were imaged. A region of interest (1 × 1.5 cm) of the graft segment was selected to determine the deposited and circulating radioactivity in each of the dynamic and static images. Radioactivity in a region of similar size of circulating radioactivity in the proximal segment of the extension tubing was determined and subtracted from the radioactivity in the graft region to calculate deposited radioactivity. 111In-platelet deposition was expressed in two ways. First, deposited graft radioactivity was expressed as a percentage of total injected 111In-platelet radioactivity. Total injected radioactivity was determined by imaging the injectate for 10 minutes. Deposited graft radioactivity was appropriately corrected for physical decay of 111In. Secondly, deposited graft radioactivity was expressed as a percentage of 111In-platelet radioactivity in the circulation at the time of imaging. This was determined from the count rate of the blood standard, corrected for plasma 111In-radioactivity.2 The corrected count rate was multiplied by the blood volume, estimated to be 65 mL/kg.22

The disappearance of 111In-labeled platelets from the circulation was also determined in four normal baboons, and the mean platelet life span calculated. The method is described in detail.17,18

**Laboratory measurements.** The times at which blood samples were collected for the different determinations can be seen in the Results section. The platelet count and hematocrit of blood samples collected in 2 mg/mL disodium EDTA were determined using a Technicon H1 blood cell analyzer (Miles Inc, Tarrytown, NY). The same blood sample was used to correct whole blood radioactivity by subtracting plasma radioactivity to calculate platelet radioactivity.13 Blood was collected in 3.8% sodium citrate (9 vol blood to 1 vol citrate) to determine activated partial thromboplastin time (aPTT), fibrinogen time (TT) and the plasma r-hirudin concentration. aPTT and TT were measured on a fibrinometer (Clotex II; Hyland Division, Travenol Laboratories, Costa Mesa, CA) using reagents supplied by the manufacturer. The plasma r-hirudin concentration was determined as described.19

**Statistical analysis.** Student’s t-test (two-tailed) for paired or unpaired data was used to test for differences when the data were normally distributed. If not, the Mann-Whitney U-test for nonparametric data was used. Data in the text are given as the mean ± SD.

**RESULTS**

**Platelet count and platelet radioactivity.** The mean platelet count before the start of the study was 352 ± 89 × 109/L in the untreated animals and 326 ± 65 × 109/L in the treated animals. The changes in platelet count following placement of the thrombogenic devices are illustrated in Fig 1A. The largest decrease, 30% (median, range = 9% to 56%) was observed in the untreated animals during the first 6 hours after placement of the devices. The corresponding decrease in the treated animals, 9% (median, range = 0 to 19%), was significantly less (P = .014, n = 14, Mann-Whitney U-test). After 6 hours, the platelet count continued to decrease in both cases until the end of the study. This decrease was similar in the untreated and treated animals, ie, 8% (median, range = 2% to 40%) and 10% (median, range = 0% to 25%), respectively (P = .844, n = 14, Mann-Whitney U-test).

The disappearance curves of 111In-platelet radioactivity from the circulation of normal baboons and in the untreated and treated baboons are shown in Fig 1B. The disappearance of labeled platelets from the circulation of both the untreated and treated animals was accelerated when compared with the untreated control.
that in the normal animals. We calculated the platelet survival time using the multiple hit model. The calculations excluded the data points collected during the first 6 hours of the study. The mean platelet life span (MPLS) of $^{111}$In-labeled platelets was normal, 138 ± 14 hours. The MPLS in the untreated animals ranged from 17 to 80 hours (median, 60 hours). In the treated animals it ranged from 35 to 79 hours (median, 55 hours). In both cases, it represented a shortening of the MPLS of approximately 40% to 43% when compared with the normal animals.

**Thrombus formation.** The results are illustrated in Fig 2A and B. Figure 2A represents the time/radioactivity curves where deposited radioactivity was expressed as a percentage of total injected radioactivity. In the untreated animals, platelets rapidly accumulated after the thrombogenic devices were placed. Maximum deposition, 1.7% ± 0.9% of injected labeled platelets, was reached approximately 4 hours after placement of the devices. Thereafter, deposition gradually decreased, reaching 0.4% ± 0.2% of the injected labeled platelets after 53 hours. Treatment with r-hirudin, started 15 minutes after the thrombogenic devices were placed (deposition = 0.3% ± 0.1% of injected radioactivity), interrupted platelet deposition during the 4 hours of treatment. In fact, it appears that deposited labeled platelets actually detached from the thrombus during treatment. After treatment was stopped, platelets were again deposited, but at a much slower rate, and to a much lesser extent than in the untreated studies. Maximum deposition, 0.7% ± 0.2% of injected labeled platelets, was reached after approximately 23 hours. Maximum deposition was approximately 59% less than in the untreated animals. Thereafter, deposition gradually decreased almost in parallel to that observed in the untreated studies. At the end of the study 0.4% ± 0.2% of the injected labeled platelets were deposited.

The results in Fig 2B represent the deposition curves when deposited radioactivity was expressed as a percentage of total circulating radioactivity at the time each image was acquired. By expressing the results in this way, one can relate the extent of deposited platelet radioactivity to the circulating radioactivity at the time of imaging. In the untreated animals, the ratio remained relatively constant after a maximum was reached. It does appear that platelets are still being deposited during the first 20 to 25 hours. Thereafter, deposited radioactivity decreased almost in parallel with circulating radioactivity, ie, the relationship remained constant. In the case of the treated animals, maximum deposition was reached approximately 23 hours after the devices were placed. Thereafter, the ratio remained relatively constant indicating that deposited radioactivity decreased in parallel with circulating radioactivity.

**Coagulation.** Figure 3A summarizes the changes in the plasma concentration of r-hirudin. When infusion was stopped, the plasma levels decreased rapidly to approximately 15% of the maximum plasma concentration 2 hours after treatment was stopped. Figure 3B summarizes the changes in aPTT in the experimental animals over the first 6 hours of the study. Ten minutes after treatment was started, the aPTT was longer than 300 seconds. This was maintained by the infusion. When infusion was stopped after 4 hours, the aPTT shortened, but did not return to pretreatment values (Table 1). There was a slight prolongation in the aPTT (significantly longer at 21 hours) in the untreated animals. The TT remained slightly prolonged for up to 30 hours in the treated animals (Table 1).

**DISCUSSION**

In the untreated animals platelets were rapidly deposited after the thrombogenic devices were placed and maximum deposition was reached after approximately 3.5 hours. Thereafter, thrombus-associated platelet radioactivity decreased towards the end of the study (Fig 2A). Sigmoidal deposition was also obtained in patients with aorta aneurysms and Dacron vascular grafts implanted for up to 10 years, although maximum deposition was reached at a later stage. This indicates that a fresh thrombus is typically more robust than an older, stabilized one. After maximum deposition was
r-HIRUDIN REDUCES POSTTREATMENT THROMBOSIS

Thrombus formation was effectively interrupted during the 4 hours of treatment of the 15-minute old thrombus (Fig 2). This is consistent with the view that r-hirudin blocks the activity of thrombin on platelets and coagulation. It also confirms the findings that r-hirudin interrupts platelet-rich, arterial-type thrombus formation induced by Dacron vascular graft material, collagen coated tubing, endarterectomized aorta segments, and following angioplasty.6,26

When treatment was stopped, platelets were again deposited (Fig 1). However, the rate of deposition was much slower and markedly less platelets were deposited than in the untreated animals. It is notable that the short-term treatment with r-hirudin reduced the maximum size of the thrombus by approximately 59% and that maximum deposition was attained at a much later stage. Also important is the finding that at 21 to 30 hours after placement of the devices, the thrombi in both untreated and treated animals were of equivalent size (Fig 2). This, and the fact that the disappearance patterns of circulating and thrombus radioactivity in both the treated and untreated animals were almost parallel after maximum deposition was reached (Fig 2B), suggest that the surface thrombogenicity was essentially the same in the treated and untreated animals after 21 hours. The comparable shortening of the MPLS in both groups (Fig 1B) further supports the finding that surface thrombogenicity is similar after 21 to 30 hours. A shortening in MPLS is generally regarded as a sensitive measure of in vivo platelet activation and increased consumption due to thrombosis.27,28

Our results strongly suggest that the effect of r-hirudin on thrombosis was transient. In spite of this, a long-term benefit of a smaller thrombus and reduced initial thrombogenicity (<21 hours) was achieved. If acute thrombosis is indeed robust, as is suggested by the deposition curves in the untreated animals, the early intervention of thrombosis could produce a lasting benefit in certain clinical settings. This was indeed the case where a 2-hour infusion of r-hirudin reduced restenosis following balloon angioplasty of atherosclerotic arteries in rabbits, possibly as a result of inhibition of platelet-dependent thrombus formation.29 These beneficial effects were obtained when treatment was started before angioplasty. In this study, we started treatment 15 minutes after the thrombogenic devices were placed. Therefore, we simulated a situation where normal hemostasis should have been achieved before treatment was started. This was done to

Table 1. Changes in aPTT and TT in the Untreated and r-Hirudin-Treated Animals

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Untreated</th>
<th>0</th>
<th>4</th>
<th>6</th>
<th>21</th>
<th>30</th>
<th>47</th>
<th>53</th>
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<td>aPTT (s)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Untreated</td>
<td>36 ± 5</td>
<td>40 ± 3</td>
<td>41 ± 4*</td>
<td>39 ± 3</td>
<td>39 ± 3</td>
<td>41 ± 8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treated</td>
<td>39 ± 3</td>
<td>&gt;300</td>
<td>168 ± 33*</td>
<td>61 ± 10*</td>
<td>56 ± 9*</td>
<td>50 ± 3*</td>
<td>50 ± 3*</td>
<td></td>
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<tr>
<td>TT (s)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Untreated</td>
<td>29 ± 2</td>
<td>30 ± 3</td>
<td>30 ± 5</td>
<td>29 ± 2</td>
<td>31 ± 5</td>
<td>30 ± 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treated</td>
<td>27 ± 1</td>
<td>&gt;180</td>
<td>&gt;180</td>
<td>35 ± 4*</td>
<td>37 ± 7*</td>
<td>28 ± 5</td>
<td>26 ± 3</td>
<td></td>
</tr>
</tbody>
</table>

Values are given as a mean ± 1 SD.

* P < .05 vs time 0, Student's t-test for unpaired data.

Fig 3. Changes in the plasma concentration of r-hirudin (A) and aPTT (B) in the treated animals. Values are given as a mean ± 1 SEM.

reached, thrombus radioactivity decreased almost in parallel with circulating radioactivity (Fig 2B). This strongly supports the hypothesis that a dynamic equilibrium exists between deposited and circulating platelets, and that the rate of thrombus formation and dissolution is equivalent. It is unlikely that the loss of radioactivity from the thrombus was due to elution of 111In from deposited platelets, because 111In does not elute from labeled platelets when they are aggregated in vitro, or, when they are incubated at 37°C for 24 hours in plasma.24,25

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reduce the risk of excessive bleeding as a result of ineffective hemostasis due to treatment. In view of our results, similar beneficial results may be expected in the maintenance of coronary artery patency following successful thrombolysis or after vascular engraftment, without adversely increasing the risk of excessive blood loss.

The mechanism by which the lasting benefit was achieved is unclear. It is unlikely that residual r-hirudin in plasma or, r-hirudin in complex with circulating thrombin, played a part. First, r-hirudin was rapidly removed from the plasma (Fig 1A), and secondly, the half-life of the effect of circulating r-hirudin on aPTT, approximately 47 minutes, was too short to affect the time to reach maximum deposition. It is possible that the characteristics of the thrombus surface played a part. r-Hirudin forms a tight complex with thrombin and can bind and inactivate thrombin bound to fibrinogen in a thrombus. In complex with thrombin, r-hirudin also inhibits the thrombin-platelet reaction. These characteristics may explain why platelet deposition was interrupted during the treatment period. The long-term benefits with respect to thrombus size may also relate to these inhibitory functions of r-hirudin. It is possible that, because of its high affinity for thrombin, the r-hirudin bound to thrombus-associated thrombin may remain bound for relatively long periods, even when no circulating r-hirudin is present. If this is the case, recruitment of platelets to the thrombus can be mediated by nonthrombin mechanisms like ADP or thromboxane A2 as r-hirudin does not affect the activity of these agonists on platelet function. This can also explain the slower rate of platelet uptake because these two platelet agonists are not as potent as thrombin.

After the rapid shortening during the first 2 hours after treatment was stopped (Fig 2B), the aPTT remained slightly, but significantly longer than pretreatment times for the remainder of the study (Table 1). It is possible that thrombin, which forms as a result of thrombogenesis, activated protein C. It was recently shown that infusion of low doses of thrombin into baboons increases the plasma levels of activated protein C in a dose-dependent manner and that aPTT lengths accordingly. This may explain the slowly prolonged aPTT at later stages of the study. This mechanism could also be responsible for the slower rate of platelet deposition and the decrease in maximum thrombus size in the treated animals. It was shown that activated protein C inhibits platelet-dependent thrombus formation.

Our results indicated that a 4-hour interruption of platelet deposition onto a freshly formed thrombus produced a lasting effect. After treatment was stopped, the rate of platelet deposition was much slower than in the untreated animals, the size of the thrombus was markedly reduced, and maximum deposition was reached at a much later stage. AV shunt patency was also affected. In the treated animals, only one shunt occluded in comparison with all the shunts in the untreated animals. These benefits were achieved despite the finding that the effect of r-hirudin was transient, and that after 21 to 30 hours, the thrombogenicity of the thrombus surfaces were similar in the treated and untreated animals. Our approach to start treatment after normal hemostasis had been achieved was justified and may be applied in certain clinical settings to reduce the risk of bleeding. This approach may be used to reduce restenosis after balloon angioplasty, to improve vascular graft patency after implantation, and to maintain coronary artery patency after successful fibrinolysis.

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REFERENCES


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