Comparison of the Antithrombotic Effect of PEG-Hirudin and Heparin in a Human Ex Vivo Model of Arterial Thrombosis

J.P. Bossavy, K.S. Sakariassen, K. Rübsamen, C. Thalamas, B. Boneu, Y. Cadroy

Abstract—Polyethylene glycol (PEG)-hirudin is a derivative of hirudin with a long plasma half-life. We have compared the efficacy of PEG-hirudin with unfractionated heparin (UH) in preventing arterial thrombosis. Arterial thrombus formation was induced ex vivo in 12 healthy human volunteers by exposing a tissue factor–coated coverslip positioned in a parallel-plate perfusion chamber to flowing nonanticoagulated human blood drawn directly from an antecubital vein at an arterial wall shear rate of 2600 s⁻¹ for 3.5 minutes. PEG-hirudin, UH, or saline (as control) were administered ex vivo through a heparin-coated mixing device positioned proximal to the perfusion chamber. Platelet and fibrin deposition was quantified by immunoenzymatic measure of the P-selectin and d-dimer content of dissolved plasmin-digested thrombi, respectively. UH was administered to a plasma concentration of 0.35 IU/mL. This concentration prolonged the activated partial thromboplastin time from 32 ± 1 seconds to 79 ± 4 seconds (P < 0.01). UH did not significantly prevent platelet deposition. However, fibrin deposition was reduced by 39% (P < 0.05). PEG-hirudin in plasma concentrations of 0.5, 2.5, and 5 µg/mL prolonged the activated partial thromboplastin time to 48 ± 2, 87 ± 4, and 118 ± 4 seconds, respectively. In contrast to UH, PEG-hirudin prevented both platelet and fibrin deposition in a dose-dependent manner with a >80% reduction at 5 µg/mL (P < 0.01). Furthermore, the plasma level of PEG-hirudin required to significantly prevent fibrin deposition (0.5 µg/mL) corresponded to a much shorter prolongation of activated partial thromboplastin time (48 ± 2 seconds) than that needed for UH (79 ± 4 seconds). Thus, our results are compatible with the view that thrombin is greatly involved in recruitment of platelets in evolving thrombi, and that PEG-hirudin is an effective agent for preventing arterial thrombosis in a human ex vivo experimental model.

Key Words: hirudin • heparin • arterial thrombosis • flow • platelets

Three types of antiplatelet drugs are currently available for the prevention and treatment of acute and chronic arterial diseases, ie, aspirin, which blocks platelet production of thromboxane A₂, thienopyridine derivatives, ticlopidine and clopidogrel, which inhibit ADP-induced platelet aggregation, and platelet glycoprotein IIb/IIIa receptor inhibitors such as abciximab (Reopro). Whereas abciximab, which totally blocks platelet aggregation, regardless of the platelet-activation agonists, is a very potent antithrombotic agent, the clinical potency of aspirin and thienopyridine derivatives is moderate. This result may be because the latter drugs interfere with only 1 of the various pathways of platelet activation. In addition, recent experimental studies have indicated that thrombin, which is not directly affected by either of these drugs, is a main mediator of arterial thrombus formation. Therefore, inhibition of thrombin generation and/or activity is an important antithrombotic strategy.

Unfractionated heparin (UH) is the most widely used anticoagulant, which catalyzes endogenous antithrombin inhibition of thrombin, and the factors Xa, IXa, and Xla. However, experimental and clinical studies indicate that UH is not very potent in interrupting this process. The relative resistance of platelet-dependent thrombus formation to UH may be caused by the inhibition of UH by platelet factor 4 released during platelet activation. In addition, steric or electrostatic hindrance of the large heparin/antithrombin complexes may reduce the access of the complex to thrombin sequestered or generated within the forming thrombus. Hirudin, a 65-amino-acid residue peptide found in medicinal leeches, is the most potent inhibitor of thrombin found in nature. It acts by forming a tight, stoichiometric complex with thrombin. Contrary to UH, newly available recombinant forms of hirudin are very effective at inhibiting arterial thrombosis in experimental models and are currently under...
clinical evaluation. One of their drawbacks is the relatively short plasma half-life when administered through an intravenous or subcutaneous route. When coupled with polyethylene glycol (PEG), the plasma half-life of the molecule is significantly prolonged. PEG-hirudin represents a promising new anticoagulant agent for the prevention and treatment of arterial thrombosis. However, recent data indicate that platelets are largely recruited into arterial thrombi by thrombus-bound thrombin rather than by soluble thrombin. Thus, it is possible that PEG-hirudin, which has a molecular mass of 17 kDa, may inhibit thrombus-bound thrombin less effectively than hirudin, whose molecular mass is 7 kDa. The aim of the present study was to determine the antithrombotic effect of PEG-hirudin in an arterial thrombosis model and to compare it with that of UH.

We have developed an ex vivo model of human arterial thrombogenesis that allows studies of acute initial thrombus formation in conditions close to those observed in humans. In this model, human nonanticoagulated blood is drawn directly from an antecubital vein of healthy volunteers over a thrombogenic surface coated on a coverslip positioned in a parallel-plate perfusion chamber. The thrombogenic surface consists of tissue factor (TF), 1 of the main relevant thrombogenic compounds present in atheromatous plaques. Blood interacts with TF at arterial wall shear rates (ie, 2600 s⁻¹). Thrombus formation is quantified by immunoenzymatic measure of platelet and fibrin deposits.

In the present study, PEG-hirudin and UH were both infused ex vivo through a heparin-coated mixing device. This mixing device allows infusion and homogeneous mixing of drugs in the bloodstream distal to the blood donor and proximal to the thrombogenic surface. We have previously investigated the antithrombotic effects of UH in this model and have confirmed the limited efficacy of UH in inhibiting arterial thrombogenesis.

Methods

Study Population

Twelve healthy volunteers were recruited by the Center for Clinical Investigation at Hôpital Purpan, Toulouse, France. They had no history or clinical sign of any disease, and did not take any medication that might affect blood coagulation or platelet function during the study period. The volunteers did not smoke on the day of the perfusion experiments. Clinical chemistry and hematological and hemostatic laboratory values were within the normal ranges. All subjects gave written informed consent to the protocol, which was approved by the local human subjects ethics committee (Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale, Toulouse).

Study Design

PEG-hirudin and UH were administered ex vivo through the heparin-coated mixing device to obtain plasma concentrations that prolong the activated partial thromboplastin time (APTT) to 45, 75, and 105 seconds for PEG-hirudin and to 75 seconds for UH (baseline APTT, 35 seconds). These plasma concentrations were chosen from calibration curves performed in vitro and they corresponded to 0.5, 2.5, and 5 μg/mL for PEG-hirudin and 2 μg/mL or 0.35 IU/mL for UH.

After being found eligible for the trial, each volunteer had 5 perfusion experiments, 2 in a first session, 2 in a second session, and 1 in a final session. These 5 perfusion experiments corresponded to 5 different drug regimens, ie, 1 control experiment where saline solution was perfused through the mixing device, 3 perfusion experiments with different doses of PEG-hirudin, and 1 perfusion experiment with UH. Because ~70 mL of blood was taken at each session, a period of 30 days was allowed between each session, to have a stable hematocrit throughout the study. The order of the 5 drug regimens was randomized.

Preparation of Tissue Factor Surface

TF surfaces were prepared as described previously. TF purified from human placenta (Thromborel, Behring) was diluted 1:133 in coating buffer (0.1 mol/L sodium carbonate, pH 9.5) and incubated with Thermawan plastic coverslips (Miles Laboratories) for 17 to 24 hours at 4°C.

Perfusion Experiments

Perfusion experiments were performed with a parallel-plate perfusion chamber at 37°C. After blood sample collection for determination of blood cell count (3 mL, K3 EDTA, Becton Dickinson), native blood was drawn directly from an antecubital vein through a 19-gauge infusion set (Ohmeda) over the TF-coated coverslip in the perfusion chamber. The blood flow rate was maintained at 10 mL/min by a peristaltic roller pump (Gilton, Model M312), corresponding to a wall shear rate of 2600 s⁻¹. Saline, PEG-hirudin, or UH was administered through a heparin-coated mixing device by a syringe pump at a flow rate of 0.2 mL/min (see below). The blood perfusion experiments lasted 3.5 minutes. However, the reactive surface was exposed to blood for 3 minutes only, because of the 0.5-minute residence time of blood in the mixing device.

Immunological Determination of Fibrin and Platelet Deposition

The coverslip with thrombotic deposits was placed in a plasmin solution (Chromogenex, 0.7 IU/mL, in TBS, pH 7.4) for 30 minutes at 37°C, for the immunoenzymatic quantification of platelet and fibrin deposition. Fibrin deposition was quantified by immunoenzymological determination of fibrin degradation products (Asserachrom D-Di, Stago) of plasmin-digested thrombi as described previously. The amount of deposited fibrin was determined directly from the levels of fibrin degradation products and expressed as micrograms of fibrin deposited per centimeter squared. Platelet deposition was quantified by measure of the specific platelet α granule membrane protein P-selectin. The plasmin-digested thrombus was centrifuged (4°C, 4300g, 15 minutes) and the pellet was dissolved in 400 μL of a lytic buffer, 3 times frozen and thawed, and then sonicated (4°C, 20 kHz) for 270 seconds. P-selectin levels were measured by immunoenzymoassay (Bender MedSystems) both in the dissolved pellet and in the supernatant of the plasmin-digested thrombus. Total number of platelets deposited was calculated by dividing the amount of P-selectin present in the thrombus by that present in nonactivated platelets of healthy blood donors (321 ± 14 ng/10⁶ platelets, n = 26). Results were expressed as number of platelets deposited per centimeter squared.

Determination of Platelet Activation and Thrombin Formation

Platelet activation and thrombin generation triggered by blood/TF interaction was determined by measuring plasma levels of β-thromboglobulin (β-TG) and thrombin–antithrombin complexes (T-AT), respectively, in blood samples (3.2 mL) collected by a syringe pump at the flow outlet of the chambers at between 3 and 3.5 minutes of perfusion. The syringes contained a mixture (0.8 mL) of platelet inhibitors and anticoagulants (sodium citrate, citric acid, theophylline, adenosine, dipyridamole, heparin, and aprotinin). The plasma concentrations of β-TG and T-AT were measured by immunoenzymoassays (Assera-β-TG, Stago, and Enzygnost-TAT, Behring, respectively).

PEG-hirudin, Heparin, and Saline Infusion

PEG-hirudin, UH, or saline was infused ex vivo through a disposable mixing device as previously described. The device consists of a helix fitted into a cylinder. The helix and the cylinder are covalently coated with heparin (Carmeda AB). A new set was used for each experiment. The mixing device was placed between the infusion set and the mixing device.
TABLE 1. Effect of Heparin and PEG-hirudin on Coagulation Times

<table>
<thead>
<tr>
<th>Heparin (IU/mL)</th>
<th>PEG-hirudin (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline 0.35</td>
<td>0.5</td>
</tr>
<tr>
<td>APTT(s)</td>
<td>0.31±0.03</td>
</tr>
<tr>
<td>ECT(s)</td>
<td>44±2†</td>
</tr>
<tr>
<td>PT(s)</td>
<td>12.2±0.2</td>
</tr>
<tr>
<td>0.5</td>
<td>0.51±0.06</td>
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<tr>
<td>48±2†</td>
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<tr>
<td>13.8±0.2</td>
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<tr>
<td>2.70±0.24</td>
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<tr>
<td>87±4†</td>
<td></td>
</tr>
<tr>
<td>62†</td>
<td></td>
</tr>
<tr>
<td>118±3†</td>
<td></td>
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<tr>
<td>5.13±0.44</td>
<td></td>
</tr>
<tr>
<td>527±62†</td>
<td></td>
</tr>
<tr>
<td>19.3±0.5*</td>
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</tbody>
</table>

Plasma levels of UH and PEG-hirudin, and APTT, ECT, and PT were measured in blood samples collected at the flow outlet of the chambers at between 1.3 and 1.9 minutes of perfusion.

Statistical comparisons of the clotting times obtained with UH or PEG-hirudin versus those obtained with saline (*P<0.05; †P<0.01).

Results

Study Population

Twelve healthy volunteers (6 males and 6 females), age 25 to 49 years (mean age, 34 years) were enrolled in the study. All volunteers completed the trial. Thrombus formation could not be determined in 1 case in which UH was infused. This was because of a technical problem during the perfusion experiment. No adverse events were reported.

Effect of Treatment on Coagulation Times

Saline, PEG-hirudin, or UH was infused through the heparin-coated mixing device to obtain plasma concentrations of 0.5, 2.5, and 5 μg/mL of PEG-hirudin, and 0.35 IU/mL of UH (Table 1). The measured plasma levels of PEG-hirudin and UH gave expected results. No heparin (<0.05 IU/mL) was present in plasma of volunteers administered with saline or PEG-hirudin, indicating the absence of any significant heparin release by the heparin-coated mixing device. UH did not prolong the ECT and PT. It prolonged the APTT from 32±1 seconds to 79±4 seconds (P<0.01). PEG-hirudin significantly prolonged APTT, ECT, and PT in a dose-dependent manner (P<0.01). At 2.5 μg/mL, the APTT prolongation was moderately, but significantly, greater than that obtained with UH (87±4 seconds versus 79±4 seconds, P<0.05). The ECT showed a steep increase, with PEG-hirudin plasma concentration rising from 44±2 seconds with saline to 527±62 seconds with 5 μg/mL PEG-hirudin.

Effect of Treatment on Thrombus Formation

The effect of saline, PEG-hirudin, or UH infusion on thrombus formation in TF-coated coverslips is shown in Figures 1 and 2. UH did not significantly prevent platelet deposition. In contrast, PEG-hirudin prevented platelet deposition in a dose-dependent manner. At 2.5 μg/mL of PEG-hirudin, the reduction was of 42% versus 13% for UH, but it was not significant, probably because of a relatively wide distribution of values. The reduction was significant only at the highest PEG-hirudin concentration (5.0 μg/mL); platelet deposition was potently reduced by >80% (P<0.01 versus saline). At this dose, PEG-hirudin was significantly more potent than UH (P<0.01).

UH prevented fibrin deposition by 39% (Figure 2, P<0.05). Fibrin deposition was prevented by PEG-hirudin in a dose-dependent manner (P<0.01). PEG-hirudin appeared...
Both UH and PEG-hirudin significantly prevented the platelet deposition. Data are mean ± 1 SEM values. \( *P < 0.05 \) and \( **P < 0.01 \), compared with saline infusion.

Fibrin deposition was measured by immunoenzymatic methods. Data are mean ± 1 SEM values. \( *P < 0.05 \) and \( **P < 0.01 \), compared with saline infusion.

Figure 2. Effect of UH or PEG-hirudin on fibrin deposition in TF-coated coverslips perfused at a shear rate of 2600 s\(^{-1}\) for 3.5 minutes. UH (HEPARIN) and PEG-hirudin (PEG) were infused ex vivo in flowing human blood through a heparin-coated mixing device to obtain plasma concentrations of 0.35 IU/mL, and 0.5, 2.5, and 5 \( \mu \text{g/mL} \), respectively. Saline infusion served as control. Fibrin deposition was measured by immunoenzymatic methods. Data are mean ± 1 SEM values. \( *P < 0.05 \) and \( **P < 0.01 \), compared with saline infusion.

Figure 3. Relation between the ECT and platelet or fibrin deposition on TF-coated coverslips. PEG-hirudin was infused ex vivo in flowing human blood through a heparin-coated mixing device. TF-coated coverslips were exposed for 3.5 minutes to nonanticoagulated blood at a shear rate of 2600 s\(^{-1}\). ECT was measured in blood samples collected at the flow outlet of the chambers at between 1.3 and 1.9 minutes of perfusion. Platelet and fibrin deposition was measured by immunoenzymatic methods.

In the present study, we show that PEG-hirudin prevents acute initial human thrombus formation under high shear stress in a dose-dependent manner. The antithrombotic efficacy of PEG-hirudin is most efficient, because both fibrin and platelet deposition were reduced by > 80% at 5 \( \mu \text{g/mL} \). This plasma concentration of PEG-hirudin may be achieved systemically by administering the molecule intravenously as a bolus injection.\(^{10}\) This potent antithrombotic effect contrasts with the relative resistance of arterial thrombus formation to UH. In a previous study, UH did not significantly prevent platelet deposition on TF-coated surfaces, even at plasma levels as high as 3 IU/mL, which prolonged the APTT to > 300 seconds.\(^{16}\) In the present study, PEG-hirudin was antithrombotic at plasma levels that resulted in a shorter APTT prolongation. Thus, for a comparable inhibition of fibrin deposition (ie, 57% and 39% reduction at 0.5 \( \mu \text{g/mL} \) and 0.35 IU/mL of PEG-hirudin and UH, respectively), the APTT was barely prolonged with PEG-hirudin (48 ± 2 seconds versus 32 ± 1 seconds with saline), whereas it was doubled with UH (78 ± 4 seconds). This plasma concentration of PEG-hirudin may be maintained systemically by once daily subcutaneous administrations.\(^{10}\) In a recent study performed in dogs, PEG-hirudin improved the rate of occluded carotid artery recanalization by thrombolytic therapy and prevented early reocclusion much more effectively than UH.\(^{15}\) Thus, PEG-hirudin appears to be an effective antithrombotic agent for the prevention and treatment of arterial thrombosis.

The model used in this study was designed to simulate clinically relevant human arterial thrombus formation by exposing a relevant thrombogenic surface to well-controlled blood flow. We used a shear rate of 2600 s\(^{-1}\), which is within

more potent than UH at the lowest tested plasma concentration (57% reduction at 0.5 \( \mu \text{g/mL} \) PEG-hirudin versus 39% for UH, \( P = \text{NS} \)) and it was significantly more potent than UH at the medium and high doses where fibrin deposition was reduced by 77% and 93%, respectively (\( P = 0.01 \)).

The antithrombotic effect of PEG-hirudin was significantly correlated to PT, APTT, ECT, and the measured plasma concentration of PEG-hirudin (Table 2 and Figure 3). However, because of interrelations between these different assays, we performed a multiple regression analysis to analyze the independence of association. Using this test, we found that the antithrombotic effect of PEG-hirudin was correlated exclusively to ECT (\( \beta = -0.54, P = 0.001 \), for platelet reduction; and \( \beta = -0.39, P = 0.02 \), for fibrin reduction).

Finally, the plasma levels of markers of platelet activation (\( \beta\)-TG) and thrombin formation (T-AT) are shown in Table 3. Both UH and PEG-hirudin significantly prevented the platelet release of \( \beta\)-TG in plasma and T-AT formation (\( P < 0.01 \)). Regarding \( \beta\)-TG release, PEG-hirudin was more potent than UH at the medium and high doses (\( P = 0.02 \)). This was also the case for T-AT formation at the highest PEG-hirudin plasma concentration (5 \( \mu \text{g/mL} \), \( P = 0.01 \)).

### Discussion

In the present study, we show that PEG-hirudin prevents acute initial human thrombus formation under high shear

| TABLE 2. Relations Between Coagulation Times and the Antithrombotic Effect of PEG-Hirudin |
|-----------------|-----------------|-----------------|
|                 | Platelet Deposition (\( \times 10^{-2} \text{cm}^2 \)) | Fibrin Deposition (\( \mu \text{g/cm}^2 \)) |
| APTT(s)         | \( r = -0.50; P = 0.002 \) | \( r = -0.37; P = 0.03 \) |
| ECT(s)          | \( r = -0.54; P = 0.001 \) | \( r = -0.38; P = 0.02 \) |
| PT(s)           | \( r = -0.48; P = 0.004 \) | \( r = -0.28; P = 0.10 \) |
| Plasma concentration of PEG-hirudin (\( \mu \text{g/mL} \)) | \( r = -0.52; P = 0.01 \) | \( r = -0.36; P = 0.03 \) |

PEG-hirudin was given to 12 healthy volunteers. TF-coated coverslips were exposed for 3.5 minutes to nonanticoagulated blood at a shear rate of 2600 s\(^{-1}\). Plasma levels of UH and PEG-hirudin, and APTT, ECT, and PT were measured in blood samples collected at the flow outlet of the chambers at between 1.3 and 1.9 minutes of perfusion. Platelet and fibrin deposition was determined by immunoenzymatic measures.
the range of those found in mildly stenosed arteries. The heparin-coated mixing device connected proximal to the perfusion chamber allows accurate control of the antithrombotic plasma concentration (Table 1) and appears therefore quite useful for defining the threshold antithrombotic dose in man. It is noteworthy, and as previously shown,16,17 that because no heparin leaks from the device, the heparin molecules coated on the mixing device do not interfere with thrombus formation occurring in the TF-coated coverslip positioned downstream. Also, the immunological quantification of platelets and fibrin allows a detailed analysis of the effect of antithrombotic molecules of the major thrombus components. Both UH and PEG-hirudin interrupted fibrin deposition more effectively than the platelet accumulation. For example, fibrin deposition was significantly prevented at the lowest tested plasma level of PEG-hirudin (ie, 0.5 μg/mL) and the potent effect of UH in preventing the platelet release of thromboxane A2.29,30 The antithrombotic efficacy of PEG-hirudin may not be inhibited by different proteins released by activated platelets. Also, hirudin is able to dissociate thrombin from platelets, because thrombin has a high shear rate, thrombi are primarily composed of platelets, but thrombin inhibitors are not effective in preventing the early platelet deposition.18,19,26 Under these conditions, the role of thrombin is overcome by those of ADP and thromboxane A2.29,30 The antithrombotic efficacy of PEG-Hirudin on thrombus-promoting surfaces of heterogeneous chemical composition remains to be determined.

Various explanations for the resistance of arterial thrombosis to heparin anticoagulation have been suggested. They include platelet activation by UH,31 neutralization of UH by heparin-neutralizing proteins as platelet factor 4 released by activated platelets,3 and the inability of heparin–antithrombin complexes to inactivate thrombus-bound thrombin.4 In this regard, it is interesting that there was a discordance between the potent effect of UH in preventing the platelet release of β-TG in plasma (Table 3) and its inability to prevent platelet thrombus formation (Figure 3). This observation may indicate that the plasma levels of β-TG are triggered by both plasma-free thrombin and thrombus-bound thrombin, and that UH does not inhibit thrombus-bound thrombin,5 whereas despite its relatively high molecular mass (17 kDa), PEG-hirudin apparently does. The superior efficacy of PEG-hirudin and other direct inhibitors of activated coagulation factors over UH may also be caused by other mechanisms. For example, PEG-hirudin may not be inhibited by different proteins released by activated platelets. Also, hirudin is able to dissociate thrombin from platelets, because thrombin has a higher affinity for hirudin than for its platelet receptor.32 Finally, it is noteworthy that because UH was infused ex vivo, the role of heparin-dependent release of endothelial TF pathway inhibitor33 was neglected in this study.

APTT is used routinely to monitor the anticoagulant effect of UH. However, this test is relatively insensitive and nonspecific for measuring direct thrombin inhibitors. A previous study showed that ECT was correlated linearly with the anti-IIa activity of hirudins.22 Ecarin is a prothrombin activator from Echis carinatus venom, which catalyzes the conversion of prothrombin to meizothrombin. We show in the present study that ECT monitoring of PEG-hirudin was much more sensitive than APTT monitoring (Table 1) and that ECT was more correlated to the antithrombotic effect of PEG-Hirudin than APTT (Table 2 and Figure 3).

In conclusion, PEG-hirudin is effective in preventing arterial thrombus formation in a human ex vivo experimental model. This result is consistent with those obtained with hirudin or other direct thrombin inhibitors, which are much more effective than heparin in preventing experimental arterial thrombosis.2 Nevertheless, used in short-term treatment,
the promise of these new molecules has not been fulfilled in clinical trials. Long-term therapy appears necessary for an optimal effect in prevention and treatment of arterial thrombosis. Therefore, because of its long plasma half-life and potent antithrombotic properties, PEG-hirudin is a promising new antithrombotic molecule.

References

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