Dose- and Shear Rate-dependent Effects of Heparin on Thrombogenesis Induced by Rabbit Aorta Subendothelium Exposed to Flowing Human Blood

Werner Inauen, Hans R. Baumgartner, Thomas Bombeli, André Haeberli, and P. Werner Straub

The effect of heparin on thrombogenesis induced by the subendothelium of rabbit aorta was investigated in 24 healthy volunteers after Intravenous Injection of different doses (0, 1000, 2500, and 5000 IU). By using an ex vivo perfusion chamber system, the interaction between flowing blood and exposed subendothelium was measured at low (50 s⁻¹), intermediate (650 s⁻¹), and high (2600 s⁻¹) wall shear rates. The low shear rate simulated blood flow in venous, the intermediate shear rate in arterial, and the high shear rate in small or stenosed arterial vessels. Deposition of fibrin, platelets, and platelet thrombi on vascular subendothelium (SE) was quantified by morphometrical and Immunological techniques. Fibrin deposition prevailed at low shear rates and was only minimal at high shear rates (30±1% vs. 1±0.4% coverage of SE with fibrin, means±SEM, p<0.001). In contrast, the interaction of platelets with SE was more intense at high compared to low shear rates, as indicated by higher platelet adhesion (5±1% vs. 4±1% coverage of SE with platelets, p<0.001) and platelet thrombus volumes (4.8±1.3 vs. 0.5±0.1 μm³/mm², p<0.001). Fibrin deposition on SE was inhibited by heparin in a dose-dependent manner and was abolished after high doses. In addition, high doses of heparin reduced the height and volume of platelet thrombi at low and intermediate wall shear rates, but no effect was found at the high shear rate. Our data show that heparin inhibits the formation of both fibrin and platelet thrombi on vascular subendothelium. The lack of effect of heparin on platelet thrombus formation at high shear rates indicates that thrombin modulates the growth rate and/or stability of platelet thrombi at low and intermediate shear rates, whereas additional factors may control platelet thrombus growth and stability at high shear conditions. (Arteriosclerosis 10:607-615, July/August 1990)

Heparin is an effective and widely used agent for the prophylaxis and treatment of thromboembolism. However, the guidelines for an optimal therapy with this antithrombotic drug have been debated for several decades, and there is still some controversy on the doses considered as effective for the prevention and treatment of thrombosis. Part of the dosage problem of heparin is due to the lack of detailed information concerning 1) the relationship between dose and antithrombotic effect and 2) the impact of local flow conditions on the efficacy of this drug.

In an attempt to bridge the gap between sophisticated test tube experiments in vitro and the complex situation in vivo, we used perfusion chambers, which expose vascular subendothelium to flowing blood directly withdrawn from the antecubital vein of human subjects. The chosen model permits a quantitative assessment of fibrin, platelets, and platelet thrombi deposited on the thrombogenic vascular surface. Variation of blood flow velocity and

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A wall shear rate of 2600 s\(^{-1}\) was produced by increasing blood flow to 40 ml/min in the small perfusion chamber. The dimensions of the chambers and calculations of the shear rates have been reported previously.\(^2,4,5\) A wall shear rate of 50 s\(^{-1}\) corresponds to flow conditions in veins, a shear rate of 650 s\(^{-1}\) to those in arteries, and a shear rate of 2600 s\(^{-1}\) to flow in small or stenosed arteries.\(^5\)

**Morphological Evaluation of Blood-Subendothelium Interactions**

Evaluation of fibrin and platelet interaction with subendothelium involved two separate microscopical screens, which were both performed on the same cross-sectional areas of the vessel segment. The proximal piece of 5 mm length was dehydrated and embedded in epoxy resin as previously described.\(^9\) Semi-thin sections \(\approx 0.8 \mu\text{m}\) in thickness were prepared, and evaluations were determined at an axial position located \(\approx 4\) mm from the proximal end of the original segment, that is, adjacent to the upstream end of the center piece used for plasmin digestion (see below). In a first screen, the interactions of fibrin and platelets with the subendothelial surface were evaluated at 10 \(\mu\text{m}\) intervals. The values were expressed as the percentages of the total number of about 1000 evaluations per cross-section, that is, the percent coverage of the subendothelial surface with fibrin and adherent platelets, respectively.\(^3,16\) The identity of the fibrin was demonstrated by electron microscopy, which showed apparent banding of 230 Å of longitudinally cut fibrin strands. In a second screen, vessel sections were projected onto the recording plate of a microprocessor-linked manual optical picture analysis system (Kontron, Zürich, Switzerland) to evaluate more precisely the dimensions of the platelet thrombi.\(^3,8\) The total cross-sectional area of deposited thrombi was determined from the perimeter of each thrombus in the projected section. Normalization of the thrombus area by the vessel circumference (perimeter of the projected vessel section) resulted in the thrombus cross-sectional area per unit length of vessel segment circumference, which corresponds stereologically to the total volume of the thrombi associated with a unit surface area of subendothelium.\(^3\) Previous studies showed that the thrombus volume calculated in this manner was closely correlated with the mass of \(^{51}\text{Cr}\)-labeled platelets deposited on the surface.\(^17\) The maximum thrombus height was defined as the mean of the three highest thrombi on the subendothelium.

**Immunological Determination of Fibrin and Fibrinogen**

A reliable method for the quantitative determination of fibrin and fibrinogen in biological material has been described.\(^18\) In brief, the center piece of subendothelium with adhering thrombotic material was immersed into 1 ml of phosphate-buffered saline. One hundred microliters of a plasmin solution (80 \(\mu\)g plasminogen activated by 3000 IU of urokinase) was added, and the vessel segment was incubated for 48 hours at 37°C. In this way, the thrombotic material was degraded to the late soluble fibrin fragments D and E. Fragment E, which is more stable, was determined by radioimmunocassay with a
Table 1. Summary of Blood Values

<table>
<thead>
<tr>
<th>Determination</th>
<th>Control</th>
<th>1000 IU</th>
<th>2500 IU</th>
<th>5000 IU</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelet count (x10⁹/µl)</td>
<td>220±7</td>
<td>219±9</td>
<td>224±10</td>
<td>224±7</td>
</tr>
<tr>
<td>Leukocyte count (x10⁹/µl)</td>
<td>5.4±0.3</td>
<td>5.4±0.4</td>
<td>5.5±0.4</td>
<td>5.9±0.3</td>
</tr>
<tr>
<td>Hemoglobin (g/100 ml)</td>
<td>14.7±0.3</td>
<td>14.3±0.4</td>
<td>14.4±0.4</td>
<td>14.5±0.3</td>
</tr>
<tr>
<td>Hematocrit</td>
<td>0.44±0.01</td>
<td>0.42±0.01</td>
<td>0.43±0.01</td>
<td>0.43±0.01</td>
</tr>
<tr>
<td>Fibrinogen (g/l)</td>
<td>1.9±0.1</td>
<td>2.0±0.1</td>
<td>2.1±0.1</td>
<td>1.9±0.1</td>
</tr>
<tr>
<td>Fibrinopeptide A (ng/ml)</td>
<td>2.9±0.6*</td>
<td>1.2±0.1</td>
<td>1.0±0.2</td>
<td>0.7±0.1</td>
</tr>
<tr>
<td>Partial thromboplastin time (sec)</td>
<td>46±1</td>
<td>57±5</td>
<td>172±13</td>
<td>&gt;300</td>
</tr>
<tr>
<td>Thrombin time (sec)</td>
<td>12.3±0.2</td>
<td>25.4±2.7</td>
<td>&gt;300</td>
<td>&gt;300</td>
</tr>
<tr>
<td>REptilase time</td>
<td>normal</td>
<td>normal</td>
<td>normal</td>
<td>normal</td>
</tr>
<tr>
<td>Heparin activity (USP-U/ml)</td>
<td>0</td>
<td>0.05±0.01</td>
<td>0.19±0.02</td>
<td>0.43±0.04</td>
</tr>
</tbody>
</table>

The values represent means±SEM. The number of evaluations was n=24 for control and heparin 5000 IU and n=12 for heparin 1000 and 2500 IU.

Blood was collected by an infusion set 20 minutes after intravenous administration of glucose 5% solution (control) and heparin doses of 1000, 2500, and 5000 IU immediately before starting with the perfusion experiments. *p<0.05 vs. heparin treatment (paired t test).

From fibrinopeptide A, fibrinogen contained within adherent platelets and nonspecifically attached to the vessel segment and the thrombus was calculated on the basis of a molecular weight ratio of 3090 for two fibrinopeptides A to 340 000 for fibrinogen (multiplication factor 110). Original fibrin in the thrombus resulted from the total fibrin and fibrinogen (calculated from fragment E) minus fibrinogen (calculated from fibrinopeptide A). Determination of fibrin...
Table 2. Effect of Wall Shear Rate and Heparin on Thrombogenesis

<table>
<thead>
<tr>
<th>Determination</th>
<th>Wall shear rate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>50 s⁻¹</td>
</tr>
<tr>
<td>Surface coverage with fibrin</td>
<td></td>
</tr>
<tr>
<td>Control (%)</td>
<td>76.0±3.4</td>
</tr>
<tr>
<td>Heparin 5000 IU (%)</td>
<td>0†</td>
</tr>
<tr>
<td>Surface coverage with platelets (platelet adhesion)</td>
<td></td>
</tr>
<tr>
<td>Control (%)</td>
<td>11.0±2.3</td>
</tr>
<tr>
<td>Heparin 5000 IU (%)</td>
<td>17.5±2.61</td>
</tr>
<tr>
<td>Platelet thrombus volume</td>
<td></td>
</tr>
<tr>
<td>Control (μm³/μm²)</td>
<td>1.2±0.2</td>
</tr>
<tr>
<td>Heparin 5000 IU (μm³/μm²)</td>
<td>0.2±0.07†</td>
</tr>
<tr>
<td>Maximum thrombus height</td>
<td></td>
</tr>
<tr>
<td>Control (μm)</td>
<td>33.3±6.2</td>
</tr>
<tr>
<td>Heparin 5000 IU (μm)</td>
<td>13.5±2.8†</td>
</tr>
<tr>
<td>Perfusion time (min)</td>
<td>5</td>
</tr>
<tr>
<td>n</td>
<td>6</td>
</tr>
</tbody>
</table>

The values represent means±SEM.

Rabbit aorta subendothelium was exposed to flowing human blood at the indicated shear rates and perfusion times. Coverage of the subendothelium with fibrin and platelets and the dimensions of platelet thrombi were determined by morphometrical methods. Maximum thrombus height indicates the mean of the three tallest platelet thrombi on subendothelium. Control indicates experiments performed 20 minutes after intravenous injection of the drug solvent (glucose 5% solution). Heparin 5000 IU Indicates experiments 20 minutes after intravenous injection of heparin.

*p<0.05, †p<0.01, ‡p<0.001 vs. control (paired t test).

and fibrinogen of both nonperfused and buffer-perfused vessel segments gave only traces of fibrin and fibrinogen.

Statistics

All values are given as means±SEM. The significance of paired and unpaired data was determined by Student’s t test. Significance levels are indicated only for the major variables of interest, namely, in the morphological data (subendothelial coverage with fibrin and platelets, thrombus dimensions), to avoid spurious significance due to comparison of multiple measurements.

Results

Blood Values

Platelet and leukocyte count, hemoglobin, hematocrit, and fibrinogen were not changed by the treatment with heparin (Table 1). Due to blood collection through the tube of an infusion set, slightly elevated values of fibrinopeptide A were found in the control experiments. Heparin led to a dose-dependent prolongation of PTT and thrombin time, whereas reptilase time was within the normal range. Heparin activity was identical in blood samples taken immediately before and after each experiment, indicating a constant activity during the whole perfusion experiment. A positive linear correlation was found between the administered heparin doses and plasma heparin activity (r=0.98, p<0.001, n=48).

Effect of Different Perfusion Times on Thrombogenesis

Figures 1 and 2 summarize the effect of different perfusion times on thrombogenesis induced by subendothelium at a shear rate of 650 s⁻¹. If the vessel segments were

Figure 3. Effect of wall shear rate and heparin on subendothelial coverage with fibrin (A) and platelets (B). Rabbit aorta subendothelium was exposed to flowing human blood at the shear rates indicated. Values are extrapolated for a perfusion time of 2 minutes. Control indicates experiments performed at 20 minutes after intravenous injection of the drug solvent (glucose 5% solution). Heparin indicates experiments at 20 minutes after intravenous injection of heparin (5000 IU). The values represent means±SEM. The number of evaluations was n=12 for 650 s⁻¹ shear rate and n=6 for 50 and 2600 s⁻¹ shear rates. *p<0.05, **p<0.01, ***p<0.001 vs. control (paired t test).
Effect of Heparin on Thrombogenesis

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Figure 4. Effect of wall shear rate and heparin on platelet thrombus dimensions. Rabbit aorta subendothelium was exposed to flowing human blood at the shear rates indicated. Values are extrapolated for a perfusion time of 2 minutes. A shows the mean height of the three tallest platelet thrombi (maximum thrombus height). B shows the morphometrically determined volume of platelet thrombi. Control indicates experiments performed at 20 minutes after intravenous injection of the drug solvent (glucose 5% solution). Heparin indicates experiments at 20 minutes after intravenous injection of heparin (5000 IU). The values represent means±SEM. The number of evaluations was n=12 for 650 s⁻¹ shear rate and n=6 for 50 and 2600 s⁻¹ shear rates. **p<0.01 (paired t test).

Exposed to normal nonanticoagulated blood (control experiments), the coverage of subendothelium with fibrin increased from 3 to 5 minutes of perfusion time, without further increase from 5 to 10 minutes (Figure 1A). In contrast, platelet adhesion (coverage of the subendothelial surface with platelets, Figure 1B), as well as the height and volume of platelet thrombi (Figure 2), showed a continuous increase up to 10 minutes of perfusion time. A positive linear correlation was found between thrombus volume and perfusion time (r=0.89, p<0.001, n=24). Fibrin coverage of subendothelium was completely abolished by heparin (5000 IU) in the experiments with 3 and 5 minutes of perfusion time, and only traces were found after an exposure time of 10 minutes (Figure 1A). The heparin-induced inhibition of fibrin formation increased the available surface for platelet-subendothelium interaction, resulting in enhanced platelet coverage (Figure 1B). At a wall shear rate of 650 s⁻¹, heparin (5000 IU) reduced the dimensions of platelet thrombi independent from exposure time (Figure 2B).

Effect of Different Shear Rates on Thrombogenesis

The effect of different shear rates on the interaction of fibrin and platelets with subendothelium is summarized in Figure 4. Since there is a linear increase of platelet thrombus dimensions with time and this also approximately holds for subendothelial coverage with fibrin and platelets during the initial 5 minutes of perfusion, the data shown in Figures 3 and 4 are extrapolated.
coverage of subendothelium with platelets was slightly increased by heparin (Figure 3B). The effect of heparin on thrombus dimensions is summarized in Figure 4. Heparin significantly reduced platelet thrombus heights (Figure 4A) and thrombus volumes (Figure 4B) at low and intermediate rates, but not at high, shear rates.

**Effect of Different Heparin Doses**

Figure 5 summarizes the dose-dependent effects of heparin on thrombogenesis. The vessel segments were exposed to human blood during 5 minutes at a shear rate of 650 s⁻¹. Compared to the control, the coverage of subendothelium with fibrin was reduced by about 90% after 1000 IU of heparin and by 97% after 2500 IU (Figure 5A). After 5000 IU, fibrin formation was abolished in all volunteers. Again, the marked inhibition of surface coverage with fibrin by heparin increased the available surface for platelet-subendothelium interaction, resulting in enhanced platelet adhesion. As shown in Figure 5B, high doses of heparin (5000 IU i.v.) reduced the height of platelet thrombi by about 30% and the thrombus volume by about 50%. The heparin dose of 2500 IU inhibited platelet thrombus formation to a much lesser extent, and no reduction was found after 1000 IU.

The results of immunologically determined fibrin and fibrinogen in plasmin-digested thrombi attached to the vessel segment are summarized in Figure 6. Immunologically determined fibrin deposition showed an identical dose-dependent inhibition by heparin as found by morphometrical methods. Fibrinogen contained within adherent platelets and nonspecifically attached to the vessel segment and the thrombus was not changed by heparin treatment. A positive linear correlation was found between morphometrical and immunologically determined fibrin deposition on subendothelium (r=0.92, p<0.001, n=48).

The representative light micrographs of the blood-subendothelium interactions from one volunteer treated with placebo (control) and different heparin doses (1000, 2500, 5000 IU) are given in Figure 7. The micrographs illustrate that heparin dose-dependently reduced deposition of fibrin (in cross-section appearing as small dots) and the dimensions of platelet thrombi on subendothelium.

**Discussion**

The results of this study demonstrate that flow conditions had a crucial impact on the composition of thrombi deposited on a de-endothelialized vascular surface. Whereas the thrombi formed at low wall shear rates (50 s⁻¹) consisted mainly of fibrin, the thrombi formed at high shear rates (2600 s⁻¹) consisted almost exclusively of platelets. The thrombi formed at intermediate shear rates (650 s⁻¹) contained significant amounts of both fibrin and platelets. These findings, which are similar to the data reported by Weiss et al.,¹ indicate an inverse relationship of fibrin deposition with shear rate and an enhanced formation of platelet thrombi with increasing shear rates. The observation that fibrin deposition prevails at low shear conditions and is drastically reduced at high shear rates may be explained by rheological factors, that is, reduced concentrations of activated coagulation proteins in the boundary layer at high shear conditions.¹⁹ Rheological factors may also be important for the observed increase of platelet-subendothelium interactions with increasing shear rates, since at high shear conditions, the platelet transport to the vessel wall and the boundary layer of a growing thrombus are enhanced.²⁰

Heparin exerted distinct dose- and shear rate-dependent effects on the interaction of fibrin and platelets with subendothelium. Whereas even heparin doses as low as 1000 IU (corresponding to approximately 15 IU/kg body weight or a plasma activity of 0.05 USP-U/ml) led to a substantial decrease of fibrin deposition, higher doses virtually abolished fibrin formation (Figures 5A and 6). These findings indicate that thrombin activity is an important determinant for the formation of fibrin thrombi because they prevail at low or venous shear rates. A major and novel finding of this study is that high doses of heparin (5000 IU) also reduce the dimensions of platelet thrombi and that this effect is confined to low and intermediate shear rates (Figure 4B). Therefore, thrombin appears to modulate the growth and/or stability of platelet thrombi at low and intermediate shear rates, whereas an alternative, perhaps physically initiated, pathway may be important for platelet thrombus formation under high shear conditions. Since the high doses of heparin, which were necessary to inhibit platelet thrombus dimensions at low and intermediate shear rates, virtually abolished fibrin deposition (Figures 5A and 5B), the lack of fibrin formation might have affected the growth and/or stability of platelet thrombi. Findings in patients with afibrinogenemia studied under identical experimental conditions, however, argue against a major role of fibrin in platelet thrombus formation, since these patients had normal platelet thrombus dimensions.⁸ To further differentiate between platelet thrombus growth and stability at high shear rates, perfusion times longer than 2 minutes would be of interest. The chambers used in this study, however, do not allow longer perfusion times at the high shear rate, since it is critical to draw a blood volume of 40 ml/min from human antecubital veins for longer periods.

The reported data on the effects of heparin on platelets are controversial. The presence of heparin has been shown to enhance, reduce, or have no influence on platelet aggregation when investigated under various in vitro conditions.²¹⁻³⁴ At first glance, our findings that high doses of heparin increase platelet adherence (Figures 1B and 5) but reduce platelet thrombus dimensions (Figures 2 and 5) appear controversial, as well. Therefore, we want to point out that the observed heparin-induced increase of platelet adherence is an effect of the substantially reduced fibrin deposition, which in turn increased the available surface for platelet-subendothelium interaction. Although our study cannot resolve the controversial in vitro findings, our results, which were obtained without additives and with minimal blood manipulation, demonstrate that high heparin doses exert an inhibitory effect on the growth and/or stability of platelet thrombi under low and intermediate shear conditions.

In our perfusion experiments, we used segments of rabbit aorta that were denuded of endothelial cells.
Therefore, the observed blood-subendothelium interactions reflect thrombogenesis on a severely damaged vessel surface. This surface is highly thrombogenic, possibly due to tissue factor and connective tissue present in the subendothelium. Although a significant loss of endothelial cells is unlikely to occur in veins, venous vascular endothelial cells have been shown to express tissue factor after stimulation. With regard to the arterial part of the vascular tree, the stimulus for activating the coagulation mechanism provided by a ruptured atherosclerotic plaque or severe atherosclerosis may appear different to that provided by our ex vivo model. However, in both instances, subendothelial connective tissue is exposed to the flowing blood, and our data at shear rates of 650 s⁻¹ and 2600 s⁻¹ might reflect thrombogenesis in arteriosclerotic vessels or at sites where hemodynamic forces cause repeated endothelial injury. In addition, a study on the effects of heparin on platelet accumulation and formation of platelet-fibrin thrombi on injured neointima of rabbit aorta showed a comparable heparin-induced inhibition of blood interaction with the vessel wall.

The clinical extrapolation of our findings is that prevention of fibrin-rich thrombi as they prevail at the low shear conditions in the venous circulation can be achieved by relatively low heparin doses. High doses of heparin are, however, necessary to inhibit thrombogenesis at the intermediate shear rates in arterial vessels. Finally, the thrombi formed at high shear rates, which consist almost exclusively of platelets, are relatively resistant against anticoagulation with heparin.

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References


Index Terms: heparin • fibrin • platelets • subendothelium • thrombosis • anticoagulation
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