Thrombin Plays a Key Role in Late Platelet Thrombus Growth and/or Stability

Effect of a Specific Thrombin Inhibitor on Thrombogenesis Induced by Aortic Subendothelium Exposed to Flowing Rabbit Blood

Alain Gast, Thomas B. Tschopp, Hans R. Baumgartner

Abstract Thrombin is involved in the pathogenesis of venous and arterial thrombosis. This study addressed the question of the relative importance of thrombin in the early and late phases of thrombogenesis. The effect of Ro 46-6240 (1.43 mg/kg bolus and 0.1 mg/kg per minute IV), a novel, selective thrombin inhibitor on thrombogenesis induced by rabbit aorta subendothelium, was measured ex vivo in a perfusion chamber model after a short (5-minute) and long (30-minute) exposure to thrombin inhibitor on thrombogenesis induced by rabbit aorta subendothelium, was measured ex vivo in a perfusion chamber model after a short (5-minute) and long (30-minute) exposure time to rabbit native blood. The role of the perfusion time was assessed at shear rates of 100/s, 650/s, and 2600/s. These shear rates mimic blood flow conditions found in veins, arteries, and small or stenosed arteries, respectively. Fibrin deposition and platelet thrombus formation on subendothelium were evaluated by microscopic morphometry. In the presence of Ro 46-6240, fibrin deposition was abolished at both perfusion times and at all shear rates. In the 5-minute experiments, thrombus height was reduced by Ro 46-6240 at shear rates of 100/s (85%) and 650/s (35%) but not at a shear rate of 2600/s, whereas thrombus area was not affected at any shear rate. In contrast, both thrombus height and thrombus area were reduced (60% to 90%) by Ro 46-6240 in the 30-minute perfusion groups at all shear rates. The antithrombotic effect of Ro 46-6240 after 30-minute perfusion was confirmed by the minimal increase in the pressure difference between the entrance and the exit of the perfusion chamber compared with the control groups. In summary, our data suggest that thrombin activity is not involved in platelet adhesion and in the initial platelet thrombus formation that occurs in early thrombogenesis but that thrombin is a major mediator of subsequent thrombus growth and/or stabilization. (Arterioscler Thromb. 1994;14:1466-1474.)

Key Words • thrombin inhibitors • thrombus formation
• shear rate • platelets • fibrin deposition

Thrombus formation at the site of vascular injury is a complex cascade of dynamic processes that involve various blood proteins such as coagulation factors (eg, thrombin) and cells, mainly platelets, erythrocytes, and leukocytes. The development of ex vivo thrombosis models by means of devices derived from the perfusion chamber originally described by Baumgartner1 has led to a more precise understanding of the relative importance of these interacting procoagulant agents in the events mediating thrombus formation. With the perfusion chamber model, it could be shown that the involvement of the different interplayers depends essentially on the thrombogenic surface and on the local shear rate conditions.2–4 For example, von Willebrand factor plays an essential role as cofactor for platelet adhesion to subendothelium at high blood shear rates but is not involved in platelet-subendothelium interactions at low shear rates.5,6 The differential effect of shear rate on thrombogenesis is also reflected in the composition of the thrombus. Thrombi formed in veins where low shear rate conditions prevail consist mainly of fibrin, whereas thrombi developed in arteries where high shear rate conditions are encountered are almost exclusively composed of platelets.7

The pivotal role of thrombin in the formation of venous fibrin-rich thrombi has been confirmed by hirudin, the most specific and potent thrombin inhibitor in animal thrombosis models and in clinical studies.8,9 In addition, recent reports suggest that thrombin may also be an important mediator in the formation of arterial platelet thrombi at high shear.10–12 The antithrombotic effects of hirudin,13 hirudin-derived peptides, the irreversible inhibitor α-Phe-Pro-Arg-chloromethyl ketone14 and heparin15 have been evaluated in an ex vivo arteriovenous shunt thrombosis model in baboons, which enables investigators to follow the time course of platelet deposition onto different thrombogenic segments under low and high shear rate conditions. In these studies, interestingly, the platelet deposition-time curves of all groups treated with anticoagulants deviated from the curves of the untreated controls only after approximately 20 minutes, suggesting that the initial phase of thrombus formation is independent of thrombin generation but that thrombin is a crucial mediator of the later phases of thrombus growth. In addition, platelets from hemophiliacs adhere normally to subendothelium, whereas fibrin deposition is completely absent, supporting the hypothesis that thrombin formation is not required for initial platelet attachment.16

To test the concept that thrombin is mainly involved in the later phases of thrombus growth, we assessed the...
antithrombotic effect of a new, synthetic, and selective thrombin inhibitor (Ro 46-6240) in the perfusion chamber ex-vivo thrombosis model after 5- and 30-minute perfusion of rabbit subendothelium with rabbit whole blood at low, intermediate, and high wall shear rates. Our findings suggest that thrombin generation is essential in the later phase of thrombogenesis for thrombus growth and/or stabilization. In addition, these results confirm that thrombin is an important mediator of acute thrombosis at low and high shear conditions.

Methods

Animals

One hundred adult Burgundy and New Zealand rabbits from different commercial sources and of either sex (weight, 3 to 4 kg) were used as blood donors and to prepare the vessel segments for the perfusion studies (see below). Before use, they were kept in quarantine for at least 2 weeks and received a normal rabbit chow (Nafag 814) and water ad libitum during this period.

Ro 46-6240

Ro 46-6240 (N-[N-[4-[[1(S)-1-amidino-3-piperidinyl]methyl]-N2-(2-naphthalenesulfonyl)-1-asparaginyl]N-cyclopropylglycine) (Fig 1) is a new, synthetic inhibitor of thrombin (K\text{,} 0.3 \text{ mmol/L}) with a high selectivity, since the inhibition constants for the other serine proteases tested are at least three orders of magnitude higher than that for thrombin (data not shown). The synthesis and the in vitro anticoagulant properties will be described elsewhere (J. Ackermann et al, unpublished data, 1994). Ro 46-6240 in the samples was calculated from a standard curve constructed with rabbit plasma containing known amounts of Ro 46-6240. The limit of detection of this assay amounts to a plasma concentration of 0.02 \text{ \mu mol/L}.

Study Protocol

Rabbits were anesthetized with sodium pentobarbital (33 mg/kg IV), and one or two additional injections were administered if necessary. The right carotid and the right femoral arteries as well as both jugular veins were then carefully dissected and canulated using silicone elastomer catheters (see below). One group of rabbits received simultaneously an intravenous bolus injection of 1.43 mg/kg Ro 46-6240 into the left jugular vein and a continuous infusion of 0.1 mg/kg per minute for 10 or 35 minutes. The drug solvent (Ringer’s lactate) was administered in the same way to a second group of rabbits (placebo control). The perfusion experiments were started 5 minutes after the administration of Ro 46-6240 or placebo and were run for 5 or 30 minutes.

Blood Values and Clotting Tests

Blood was collected from a catheter inserted into the right femoral artery immediately before the start of each perfusion experiment and at three time points during the perfusion. Platelet, red blood cell, and leukocyte counts; hemoglobin; and hematocrit were evaluated in EDTA blood by an electronic counting device (Contraves Digicell 800, Contraves Medical). At the same time points, blood was collected on 108 mmol/L trisodium citrate; 9 parts of blood to 1 part of citrate solution and platelet-poor plasma was obtained after centrifugation at 10 000 g for 2 minutes at room temperature in an Eppendorf centrifuge. Activated partial thromboplastin time (partial thromboplastin time reagent, Roche) and thrombin time (bovine thrombin reagent, Roche; final concentration, 6 U/mL) were performed with citrated plasma by means of a coagulation timer (Fibrometer, Becton-Dickinson).

Plasma Concentration of Ro 46-6240

The same citrated plasma samples were also used to measure the concentration of Ro 46-6240. Briefly, 200 mL plasma was mixed with 400 \text{ \mu L} acetone and incubated at least 5 minutes at room temperature. After centrifugation at 12 000 g for 2 minutes at room temperature, 500 \text{ \mu L} of the supernatant was evaporated under nitrogen. The evaporate was resuspended in 167 \text{ \mu L} of 0.05 mol/L tris(hydroxymethyl)aminomethane (Tris), 0.1 mol/L NaCl, 0.1% (wt/vol) polyethylene glycol 6000, and 0.02% (vol/vol) polysorbate 80, pH 7.8 (equivalent to 200 \text{ \mu L} plasma thrombin inhibitor activity of the solution was measured with a chroomogenic substrate kinetic assay run on a Cobas Bio spectrophotometric centrifugal analyzer (Roche Diagnostica). The final concentration of human thrombin was 12 \text{ nmol/L}, and that of the substrate synthetized in-house, methoxysulfonyl-D-Leu-Gly-Arg-paranitroanilide, was 100 \text{ \mu mol/L}. The concentration of Ro 46-6240 in the samples was calculated from a standard curve constructed with rabbit plasma containing known amounts of Ro 46-6240. The limit of detection of this assay amounts to a plasma concentration of 0.02 \text{ \mu mol/L}.

Preparation of Subendothelium Segments

The aortas of anesthetized rabbits were excised, mounted on a small plastic rod, and everted as described previously.17 This procedure leads to a complete remucosalized endothelium. The aortas were then cut into segments of approximately 15 mm and stored at 4°C for up to 3 weeks in 0.2 mol/L Tris (pH 7.4) until use.

Perfusion Procedure

An annular perfusion chamber was used to expose rabbit subendothelium to rabbit whole blood under controlled flow conditions. Briefly, native blood was circulated for 5 or 30 minutes from a carotid artery via silicone elastomer tubing through the annular space formed by the outer cylinder of the perfusion chamber and the vessel segment mounted on the rod. The blood returned to the general circulation via silicone elastomer tubing inserted in the right jugular vein. A jacket containing the chamber was connected to a water bath maintained at 38°C. Variable flow rates and chamber dimensions were used to mimic venous and arterial blood flow conditions. The desired flow rate was controlled by a roller pump (Perpex Jubile). The blood flow rate as well as the pressure at the entrance and exit of the perfusion chamber was monitored by an electromagnetic flow meter (Hellige Instruments, Rüegg Medical). A constant blood flow rate of 20 mL/min resulted in shear rate of 100/s at the vessel surface of 100% in the large chamber. Blood flows of 10 and 40 mL/min in the small chamber resulted in wall shear rates of 650/s and 2600/s, respectively. The dimensions of the chambers and calculations of the shear rates have been reported previously.16,18,19

Fixation and Processing of the Subendothelium Segments

Immediately after perfusion, the vessel segment still mounted on the rod was rinsed with phosphate-buffered saline and fixed with 2.5% glutaraldehyde/0.1 mol/L cacodylate buffer under perfusion conditions without flow cessation. The rod with the segment was then removed from the chamber, postfixed with glutaraldehyde, and embedded in epoxy resin as described.20

FIG 1. Chemical structure of Ro 46-6240.
**Morphological Evaluation of Blood-Subendothelium Interactions**

Platelet-surface and fibrin-surface interactions were differentiated and quantified with (1) standard morphometry and (2) computer-assisted morphometry at an axial position of 1 mm at the proximal end of the exposed segment in semithin sections of 0.8-μm thickness cut perpendicular to the direction of the blood flow.

(1) Standard morphometry was used to quantify percent surface coverage with adherent platelets (contact and spread platelets) and with fibrin. The evaluations were carried out at ×1000 magnification with light microscopy (Planapo 100/1.3, Zeiss) at 10-μm intervals. The values were expressed as the percentages of the total number of approximately 1000 evaluation points per cross section.

(2) Computer-assisted morphometry, an IBM-compatible computer, and the DIASYS program (Heinz Meyer, DataLab) were used to quantify thrombus area (μm²/μm). The maximum thrombus height (μm) was defined as the mean of the three highest thrombi. The thrombi present directly on the surface and above the subendothelium were evaluated.

**Statistical Analysis**

All values are expressed as mean±SEM. The significance of the differences between control and Ro 46-6240-treated groups was assessed with the two-sided Mann-Whitney U test. The statistical analysis was performed with the analysis package of the RS/1 data handling system (BBN Software Products Corp). The statistical significance was defined as *P<0.05, †P<0.01.*

**Effect of Perfusion Time and Shear Rate on Thrombogenesis**

The plasma concentrations of Ro 46-6240 measured at the beginning, middle, and end of the 5- and 30-minute perfusion experiments were similar and therefore were averaged. The plasma concentration of Ro 46-6240 at steady state was up to 9 μmol/L and on average was approximately 20% higher in the 30-minute perfusion experiments compared with the 5-minute experiments (Table 2). This increased plasma concentration translated into higher activated partial thromboplastin time values. The thrombin time was more than 200 seconds in all rabbits treated with this dose of Ro 46-6240, indicating full anticoagulation (Table 2).

**Results**

**Blood Values**

We calculated the mean values of the hematologic and coagulation variables of the control and drug-treated groups according to the perfusion time, since there was no difference between the drug-treated groups at the three shear rates tested (data not shown). A slight decrease of all blood variables measured was noticed in both control and drug-treated groups at the end of the 5- and 30-minute perfusion experiments (Table 1). The postperfusion values were approximately 5% to 15% lower than the baseline values and remained in a normal range. The leukocyte count, which was the most affected parameter, decreased by approximately 30% to 50% in both the control and Ro 46-6240-treated groups.

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**Table 2. Plasma Concentration and Effect of Ro 46-6240 on Coagulation**

<table>
<thead>
<tr>
<th>Determination</th>
<th>5-Minute Perfusion</th>
<th>30-Minute Perfusion</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Control (n=30)</td>
<td>Ro 46-6240 (n=15)</td>
</tr>
<tr>
<td>Plasma concentration, μmol/L</td>
<td>0.0</td>
<td>6.5±0.5</td>
</tr>
<tr>
<td>aPTT, s</td>
<td>19.9±0.5</td>
<td>75.8±3.3†</td>
</tr>
<tr>
<td>TT, s</td>
<td>5.7±0.1</td>
<td>&gt;200</td>
</tr>
<tr>
<td>n</td>
<td>30</td>
<td>15</td>
</tr>
</tbody>
</table>

*aPTT indicates activated partial thromboplastin time; TT, thrombin time; and n, total number of rabbits in each group. Values are mean±SEM of three plasma samples taken 0, 2.5, and 5 minutes after start of the 5-minute perfusion experiments and 0, 15, and 30 minutes after start of the 30-minute perfusion experiments. The aPTT values of the drug-treated groups correspond to a 3.8- and 6.4-fold prolongation of the baseline values in the 5- and 30-minute perfusion groups, respectively. The TT values in the drug-treated groups were prolonged more than 34-fold compared with baseline value in both the 5- and 30-minute perfusion experiments.

*P<0.05, †P<0.01, 5- vs 30-minute perfusion values (unpaired Mann-Whitney test).
TABLE 3. Effect of Perfusion Time and Ro 46-6240 on Thrombogenesis

<table>
<thead>
<tr>
<th>Determination</th>
<th>Wall Shear Rate, s⁻¹</th>
<th>5</th>
<th>100</th>
<th>30</th>
<th>650</th>
<th>5</th>
<th>30</th>
<th>2600</th>
<th>5</th>
<th>30</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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</tr>
<tr>
<td>Surface coverage with fibrin, %</td>
<td>Control</td>
<td>85.1±3.4</td>
<td>66.2±3.1</td>
<td>35.4±8.4</td>
<td>15.4±4.7</td>
<td>13.5±3.0</td>
<td>0.9±0.9</td>
<td>Ro 46-6240</td>
<td>0.0±0.0t</td>
<td>0.1±0.1*</td>
</tr>
<tr>
<td>Surface coverage with platelets (platelet adhesion), %</td>
<td>Control</td>
<td>6.8±0.7</td>
<td>22.7±3.7</td>
<td>29.1±3.8</td>
<td>82.6±5.1</td>
<td>74.9±2.1</td>
<td>98.3±0.8</td>
<td>Ro 46-6240</td>
<td>15.8±1.3t</td>
<td>82.5±11.4*</td>
</tr>
<tr>
<td>Platelet thrombus area, μm²/μm</td>
<td>Control</td>
<td>0.4±0.1</td>
<td>18.9±3.4</td>
<td>7.5±6.8</td>
<td>113.9±18.5</td>
<td>56.9±5.5</td>
<td>14.0±0.1t</td>
<td>Ro 46-6240</td>
<td>0.2±0.1</td>
<td>3.0±2.2*</td>
</tr>
<tr>
<td>Maximum thrombus height, μm</td>
<td>Control</td>
<td>78.8±10.7</td>
<td>276.3±30.7</td>
<td>121.0±12.4</td>
<td>379.1±51.4</td>
<td>218.0±12.9</td>
<td>195.0±62.6</td>
<td>Ro 46-6240</td>
<td>12.5±3.4†</td>
<td>34.7±13.3*</td>
</tr>
<tr>
<td>No. of animals tested (n)</td>
<td>Control groups</td>
<td>13</td>
<td>6</td>
<td>5</td>
<td>9</td>
<td>12</td>
<td>2</td>
<td>Ro 46-6240-treated groups</td>
<td>5</td>
<td>3</td>
</tr>
</tbody>
</table>

Values are mean±SEM of n observations. Rabbit aorta subendothelium was exposed to flowing rabbit 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 morphometric methods. Maximum thrombus height indicates the mean of the three tallest platelet thrombi. Control indicates experiments performed after intravenous administration of the drug solvent. Ro 46-6240 indicates experiments performed after intravenous administration of Ro 46-6240 (1.43 mg/kg bolus and continuous infusion of 0.1 mg/kg per minute).

*P<0.05, †P<0.01 vs control.

†No exact values were measurable because growth in height was limited by the outer wall of the perfusion chamber. Therefore, thrombi touched the outer wall of the chamber and were disrupted and partially lost during removal of the rod from the chamber.

seen in Table 3 and Fig 2. Platelet deposition on subendothelium increased and fibrin deposition decreased with both shear rates and perfusion times (Table 3, Fig 2). At a wall shear rate of 2600/s, fibrin coverage was approximately 14% after 5-minute perfusion, whereas only traces of fibrin were visible (approximately 1% coverage) after 30-minute perfusion, probably because additional fibrin was masked by platelet masses.

In contrast to fibrin, platelet adhesion increased with shear rate (Table 3), and the dimensions of platelet thrombi, indicated as thrombus height and thrombus area, were also enhanced at high shear conditions (Table 3, Fig 2). Thrombus height was approximately threefold higher after 30-minute than after 5-minute perfusion, whereas thrombus area increased approximately 40- and 10-fold at shear rates of 100/s and 650/s, respectively. It should be noticed that precise values of thrombus height and thrombus area could not be obtained for the 30-minute perfusion time experiments at a shear rate of 2600/s, since growth in height was limited by the annular width (350 μm) of the small perfusion chamber, ie, the distance between the inner wall of the cylinder of the chamber and the subendothelium surface.24 Therefore, thrombi touched the outer wall of the chamber and were disrupted and partially lost during removal of the rod from the chamber. In addition, only two of the four control experiments performed at a shear rate of 2600/s and 30-minute perfusion time could be morphometrically evaluated as a result of squeezing of the vessel segments caused by the complete occlusion of the annular space by thrombi.

To have an additional although indirect indicator of the thrombotic mass present in the annular space of the chamber, we measured the pressure difference between the entrance and the exit of the perfusion chamber (Fig 3). The pressure difference gradually increased during the 30-minute observation period in all control groups, indicating that the pressure at the exit of the chamber decreased. The increase of the pressure difference was shear rate dependent and reached a maximal value of approximately 250 mm Hg at a shear rate of 2600/s (Fig 3). Thus, the positive correlation observed between the thrombus dimensions and the pressure differences indicates that the pressure at the exit of the chamber is related to the amount of thrombotic material formed at the surface of the subendothelium.25

Effect of Ro 46-6240 on Thrombogenesis After 5- and 30-Minute Perfusion

The administration of Ro 46-6240 completely abolished the fibrin deposition on subendothelium for both perfusion times at all three shear rates (Table 3, Fig 2), indicating that the procoagulant activity of thrombin.
At time of shear rate of 2600/s, whereas the pressure difference remained essentially unchanged at shear rates of 100/s and 650/s.

In the 5-minute experiments, only thrombus height but not thrombus area was reduced by Ro 46-6240 at shear rates of 100/s (85%) and 650/s (35%). In contrast, both thrombus height and thrombus area were reduced (60% to 90%) by Ro 46-6240 in the 30-minute perfusion groups at all wall shear rates (Table 3, Fig 2). The pressure at the entrance and exit of the perfusion chamber remained constant over the 30-minute period (Fig 3). A slight increase compared with the baseline observed at a shear rate of 2600/s, whereas the pressure difference remained essentially unchanged at shear rates of 100/s and 650/s.

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Light micrographs representative of the blood-subendothelium interactions from rabbits treated with placebo (control) and Ro 46-6240 after 5- and 30-minute perfusion at a shear rate of 650/s are shown in Fig 4. Ro 46-6240 completely abolished deposition of fibrin after 5- and 30-minute perfusion (Fig 4B and 4D). The thrombi formed after 5-minute perfusion in the control group are high and slim and have an irregular shape, with a narrow base of attachment to subendothelium (Fig 4A). In contrast, the thrombi formed in the presence of Ro 46-6240 are lower, with a larger base of attachment to subendothelium. Furthermore, the overall shape of the thrombi is more regular than that of the thrombi in the control group (Fig 4B). The thrombi after 30-minute perfusion (control) form a compact mass occupying nearly all the annular space (Fig 4C). In the presence of Ro 46-6240, the thrombotic mass is significantly reduced and the thrombi present the same broad surface of contact and are hilly-shaped, as already observed in the 5-minute experiments (Fig 4D).
Discussion
Numerous animal studies with specific thrombin inhibitors have shown that thrombin is an important mediator in the formation of venous and arterial thrombi. However, these thrombosis models used end-point measurements to assess the overall antithrombotic effect of the tested anticoagulants. Therefore, these studies give no insight into the time course of events in which thrombin may be involved.

We used the well-established ex vivo perfusion chamber thrombosis model in rabbits to assess the relative importance of thrombin in the early and late stages of a growing thrombus. To this purpose, we compared the effect of the new, selective thrombin inhibitor, Ro 46-6240, after a short (5-minute) and long (30-minute) perfusion time of rabbit aorta subendothelium with whole nonanticoagulated native blood. The perfusion time dependence of the antithrombotic effect of Ro 46-6240 was evaluated at wall shear rate conditions found in veins (100/s), arteries (650/s), and stenosed arteries (2600/s).

The results of this study confirm the observations made by Weiss et al and Inauen et al that flow condition is a major parameter governing the composition of thrombi built up on a de-endothelialized vessel surface. The thrombi formed at low wall shear rates (100/s) consisted mainly of fibrin, the thrombi formed at intermediate shear rates (650/s) were composed of significant amounts of both fibrin and platelets, and the thrombi formed at high shear rates (2600/s) contained almost exclusively platelets (Table 3). These findings indicate an inverse relation of fibrin deposition with shear rate and an enhanced proportion of platelets in thrombi with increasing shear rate, as already shown by Weiss et al. In addition, we show that this observation holds for both short and long exposure times (5 and 30 minutes). Rheological factors might explain these results because at high shear conditions (1) the concentrations of activated coagulation factors in the boundary layer are reduced and (2) the platelet transport to the vessel wall and the boundary layer of a growing thrombus are enhanced.

Ro 46-6240, a reversible and competitive inhibitor of thrombin, exerted distinct perfusion time- and shear rate-dependent effects on fibrin deposition and thrombus formation on subendothelium. Ro 46-6240 at a fully anticoagulant dose abolished fibrin formation at both perfusion times and at all shear rates (Table 3, Fig 2). Maximal reduction of thrombus dimensions were observed after 30-minute perfusion at a shear rate of 100/s (84% reduction of thrombus area and 87% reduction of thrombus height) (Table 3, Fig 2). These results are in agreement with earlier studies showing that thrombin activity is a major determinant for the formation of fibrin-rich thrombi occurring at low or venous shear rates. Tissue factor exposed on the subendothelium is a likely candidate for the initiation of thrombin generation and subsequent fibrin deposition on the subendothelium surface.

The complete inhibition of fibrin deposition by Ro 46-6240 did not preclude thrombus formation on subendothelium after 30-minute perfusion (Table 3, Fig 2), suggesting that thrombin generation and fibrin deposition are not prerequisites for platelet attachment and early platelet thrombus growth. This view is strengthened by the fact that one patient with afibrinogenemia studied in the perfusion chamber thrombosis model had normal platelet thrombus dimensions.

Ro 46-6240 significantly reduced the thrombus height after 5-minute perfusion at shear rates of 100/s and 650/s, but this decrease was not accompanied by a parallel reduction of the thrombus area (Table 3). This observation is explained by the fact that the thrombi formed in absence of Ro 46-6240 (control) are tall and irregularly shaped, with "overhanging" structures, whereas those formed in presence of Ro 46-6240 are comparatively low and hill-shaped (Fig 4A and 4B). Thus, although the overall thrombus dimensions were not reduced by Ro 46-6240, qualitative differences in the morphology of the thrombi exist. Interestingly, studies comparing native human blood with citrated blood after short perfusion times (2 to 3 minutes) at intermediate and high shear conditions have shown the same qualitative differences in the shape of the thrombi and their base of attachment on the subendothelium. The absence of a significant antithrombotic effect of Ro 46-6240 after 5-minute perfusion is in accordance with the results of Baumgartner, which showed that heparin (500 U/kg) completely inhibited fibrin deposition but had no effect on platelet thrombus formation after 3-minute perfusion of rabbit aorta subendothelium with rabbit native blood at a shear rate of 1300/s. Another study at a shear rate of 650/s and with the same dose of heparin confirmed that heparin has no effect on platelet thrombus formation after 3-minute perfusion but showed that heparin reduced thrombus dimensions after 5-minute perfusion. However, this effect of heparin could be independent of thrombin and perhaps due to the inhibition of other coagulation factors such as factor VIIa or Xa. A recent study showed that heparin given at high doses (2500 and 5000 U/kg) to human volunteers reduced thrombus formation at low (50/s) and intermediate (650/s) but not at high (2600/s) shear conditions after short exposure times (3 to 10 minutes) of rabbit subendothelium to flowing human blood. The apparent discrepancy with our results could be related to the high doses used and to species differences as well as to effects of heparin independent of thrombin, as already mentioned.

A major finding of this study is that Ro 46-6240 significantly reduces the dimensions of thrombi after a long perfusion time (30 minutes) at low (100/s), intermediate (650/s), and high (2600/s) shear rates (Table 3). The slightly lower plasma concentration of Ro 46-6240 in the 5-minute perfusion groups compared with the 30-minute perfusion groups (Table 2) does not account for the lack of effect in the 5-minute perfusion experiments since Ro 46-6240 at a fivefold higher dose (0.5 mg/kg per minute; plasma concentration, 45 μmol/L) did not reduce thrombus area and thrombus height after 5-minute perfusion at a shear rate of 2600/s (data not shown).

It is interesting to note that a similar drop of the platelet count was observed in the control and Ro 46-6240-treated groups after 5-minute exposure, whereas Ro 46-6240 prevented the decrease of the platelet count in the 30-minute perfusion experiments (Table 1). This observation shows that the effect of Ro 46-6240 on platelet consumption due to thrombus for-
formation correlates with the morphologically evaluated antithrombotic activity.

The time dependence of the antithrombotic effect of selective thrombin inhibitor by Ro 46-6240 suggests that only the later phases of platelet thrombus growth involve thrombin activity, whereas the early phases, that is, the primary adherence of platelets to subendothelium, do not require thrombin generation. In contrast, whereas fibrin deposition is completely absent. Furthermore, several studies have demonstrated the pivotal role of platelet glycoproteins Ib-IIIa and Ib-IX in mediating platelet adhesion to subendothelium at intermediate and high shear conditions.

In contrast, the clear antithrombotic effect of the selective thrombin inhibitor Ro 46-6240 observed after 30-minute perfusion at all shear rates (Table 3, Fig 2) suggests that thrombin is a major participant in the modulation of thrombus growth and/or stability under venous and arterial shear rate conditions. Interestingly, in the presence of Ro 46-6240, thrombus area and thrombus height after 30-minute perfusion were similar to those measured in the control of the 5-minute perfusion experiments (Table 3, Fig 2), suggesting that no further thrombus growth occurred and/or that some thrombi were dislodged. Moreover, previous studies with heparin in the same perfusion chamber model have shown that the antithrombotic effect of heparin increases with increasing perfusion times up to 20 minutes at a shear rate of 650/s. In agreement with these observations, indicating that the main effect of thrombin is not on the initial growth of platelet thrombi but on their stability. In addition, our observation is consistent with results obtained in an ex vivo thrombogenesis model in baboons, showing that specific thrombin inhibitors have an effect on platelet deposition only after approximately 20 minutes of exposure time.

Based on the results of this study and earlier findings, we propose the following sequence of the events governing the formation of thrombosis on exposed subendothelium. In the first phase, platelets adhere rapidly to the surface of the subendothelium, become activated, and spread, and initial platelet thrombus growth starts; this process appears independent of thrombin. In the second phase, the activated platelets constitute a procoagulant surface where high concentrations of thrombin are generated locally. The formed thrombin then triggers further thrombus growth by activating circulating platelets and probably stabilizing the growing thrombi as observed ex vivo with citrated blood and in vivo in rabbits in the same system. In addition, thrombin bound to the thrombus catalyzes the generation of the fibrin network, thereby further stabilizing the platelet thrombi. Furthermore, other agents released in part by activated platelets can contribute to the proaggregatory activity of activated platelets, such as ADP and thromboxane A2, and this may explain why thrombus formation is not totally abolished by a specific thrombin inhibitor even at a high dose.

In conclusion, this study demonstrates that platelet adhesion and the initial phase of platelet thrombus growth do not require thrombin generation. In contrast, thrombin is essential for late thrombus growth and/or stability under venous and arterial shear rate conditions in our ex vivo thrombogenesis model.

Acknowledgments

We wish to thank Christine Michael, Käthi Schietinger, Heidi Hoffmann Maiochi, and Robert Ecaert for expert technical assistance; Hedi Jenny for secretarial help in preparing the manuscript; and Drs D. Kirchhofer and S. Roux for their careful reading of this manuscript and suggestions.

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Thrombin plays a key role in late platelet thrombus growth and/or stability. Effect of a specific thrombin inhibitor on thrombogenesis induced by aortic subendothelium exposed to flowing rabbit blood.

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