Effects of tirofiban on haemostatic activation \textit{in vitro}$^\dagger$


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Background. Thrombin plays a critical role in normal haemostasis and pathological thrombosis. Heparin has long been a mainstay choice of antithrombotic regimen in cardiac patients, but persistent thrombin generation seems to occur during heparin therapy. Because platelets are integral to primary haemostasis and clot formation, we evaluated the use of tirofiban (Aggrastat$^\dagger$), a platelet inhibitor, as a therapy to improve heparin sensitivity and delay thrombin formation.

Methods. Blood samples were obtained from healthy subjects ($n=8$) and cardiac surgical patients ($n=34$). Thrombin formation was measured in platelet-rich plasma with a Thrombogram$^\dagger$-Ascent fluorescent plate reader system. Platelet inhibition by tirofiban was evaluated with Plateletworks$^\dagger$, and the interaction of tirofiban and heparin (>1.5 U ml$^{-1}$) on clot formation was evaluated with Sonoclot Analyzer$^\dagger$ or kaolin activated clotting times (ACTs).

Results. Addition of tirofiban (70–280 ng ml$^{-1}$) progressively delayed onset of thrombin generation triggered by adenosine diphosphate (ADP). Plateletworks showed platelet inhibition with tirofiban (>35 ng ml$^{-1}$), whereas heparin 	extit{per se} failed to produce platelet inhibition at 7 U ml$^{-1}$. Heparin (1.5 U ml$^{-1}$) slowed the onset and rate of fibrin formation on Sonoclot analyses, and this was further slowed after addition of tirofiban (70 ng ml$^{-1}$) to heparin-containing blood samples. Significant increases in ACT at all heparin concentrations were observed with the addition of tirofiban (70 ng ml$^{-1}$). The addition of antithrombin (0.2 units/ml) to heparinized blood samples further prolonged ACTs, but the difference was not statistically significant when compared with heparin alone.

Conclusion. Tirofiban delays platelet activation-mediated thrombin generation and prolongs ACT in heparinized blood.

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Thrombin plays a critical role in normal haemostasis and pathological thrombosis. Heparin has long been a mainstay choice of antithrombotic regimen in cardiac patients for a variety of indications, such as unstable angina, coronary interventions and cardiopulmonary bypass (CPB) procedures. One of the shortcomings of heparin is its need for cofactors. Heparin resistance due to acquired antithrombin (AT) deficiency is a growing problem, especially in patients with unstable angina who are treated with unfractionated heparin that is continued until cardiac catheterization, intervention or cardiac surgery.$^1,2$ Another problem with heparin may be attributed to its inability to suppress platelet aggregation.$^3$ Recently, antiplatelet therapy using glycoprotein (GP) IIb/IIIa blockers has been successfully added to heparin anticoagulation for angioplasty and acute coronary syndrome, and this leads to improved outcomes.$^4$ Anticoagulation with tirofiban added to unfractionated heparin has also been used for cardiopulmonary bypass surgery to attenuate activation of haemostatic/inflammatory system and in patients with heparin-induced thrombocytopenia.$^5,6$ Furthermore, tirofiban given before surgery to patients requiring urgent or emergency coronary artery bypass grafting reduced haemostatic product transfusion without increasing post-operative chest tube drainage.$^7$ Because a small amount of thrombin activates platelets and cleaves fibrinogen, and the

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forming clot supports explosive thrombin formation,8 we have hypothesized that the adjunctive use of platelet inhibitors to high-dose heparin may further reduce thrombin formation. Initially we examined the effects of tirofiban on thrombin generation and platelet aggregation. We then tested the effects of combined heparin and tirofiban using whole blood clotting tests.

Materials and methods

Drugs and buffers

Adenosine-5’-diphosphate (ADP; Chronolog Corp, Haverton, PA, USA) was dissolved in normal saline (1.0 ml) and then diluted to a concentration of 0.5 mM with HEPES working buffer (pH 7.35, HEPES 20 mM, NaCl 140 mM, BSA 5 mg ml−1, Sigma, St.Louis, MO). Tissue factor (Innovin; Dade Behring, Merburg, Germany) was dissolved in 10 ml sterile water and then further diluted with working HEPES buffer (1:75).

For the preparation of Fluca buffer, HEPES buffer 1.75 ml (pH 7.35, HEPES 20 mM, BSA 60 mg ml−1) was added to CaCl2 1 M 0.2 ml (Sigma) in a glass test tube, mixed, and warmed for a few minutes at 37°C. Just before use, fluorogenic substrate 50 μl (Z-GGR-AMC, Bachem Switzerland) DMSO 100 mM (Sigma) was added to the HEPES-CaCl2 solution and mixed to dissolve. This buffer now contained substrate 2.5 mM and CaCl2 100 mM. Tirofiban (Aggrastat®; Merck, Whitehouse Station, NJ, USA), AT (Thrombate III™; Bayer, Elkhart, IN, USA) and porcine sodium heparin (Elkins-Sinn, Cherry Hill, NJ, USA) were diluted with saline and then added to platelet-rich (PRP) or whole blood samples to obtain appropriate drug concentrations. The volumes of drugs added to PRP or whole blood resulted in <1% dilution.

Thrombin generation and platelet aggregation

The study was performed after institutional approval and with informed consent.

Blood samples were collected from eight volunteers in 3.2% sodium citrate tubes. Plasma was centrifuged (5 min at 800 g) to obtain PRP, and platelet-poor plasma (PPP) (15 min at 2000 g). PPP was used to adjust the platelet count of PRP to 200 000 ml−1. Thrombin generation was measured on the basis of the development of the fluorescent signal over time,9 by using a slow fluorogenic thrombin substrate and a thrombin calibrator. Briefly, for the thrombin generation experiments, PRP 80 μl and the thrombin generation trigger (20 μl of ADP or tissue factor) were added to wells of 96-well solid black microtitre plates (Microfluor 2; ThermoLabsystems, Franklin, MA, USA) followed by substrate–calcium chloride buffer 20 μl. The reaction was monitored using microplate fluorometer (Fluoroscan Ascent; ThermoLabsystems, Franklin, MA, USA) set at 390 nm (excitation) and 460 nm (emission). Fluorescence was recorded every 20 s for 60 min and the acquired data were processed for thrombin generation parameters: lag time and endogenous thrombin potential (ETP) (Synapse, Maastricht, The Netherlands). The area under the curve (the ETP) represents the amount of thrombin enzymatic action that can potentially be triggered in a given plasma sample before its inhibition by physiological antithrombins (AT, α2-macroglobulin).10 11

The contribution of platelets to thrombin generation was assessed by adding different concentrations of tirofiban to PRP (plasma tirofiban 0, 100, 200 and 400 ng ml−1; final concentration 0, 70, 140 and 280 ng ml−1 respectively) or porcine heparin (plasma heparin 0, 0.1, 0.2 and 0.3 U ml−1; final concentration 0, 0.07, 0.14 and 0.21 U ml−1 respectively). For aggregation studies, blood was collected from eight normal volunteers in 3.2% sodium citrate (9:1 vol/vol) or sodium heparin (final concentration 7 U ml−1). Two different anticoagulants were used to verify that neither anticoagulant on its own had any aggregation effects. All the measurements were obtained within 40 min after blood sampling. For each sample, platelet inhibition by tirofiban was tested before and after in vitro addition of tirofiban (final concentration 0–140 ng ml−1), using Plateletworks (Helena, Beaumont, TX, USA) in ADP test tubes (20 μM). The instrument works as a cell counter. In the presence of an agonist (i.e. ADP) platelets aggregate, thus exceeding the threshold limitations for platelet size, and are therefore excluded from being counted as individual platelets. The differential platelet count between two samples (baseline and ADP) is used to calculate the percentage of aggregation.

Whole blood clot formation

We also evaluated the antithrombotic effects of tirofiban alone and in combination with heparin, and compared these with the effects of AT, which is known to augment heparin anticoagulation, using an in vitro whole blood clot protocol. Blood was collected from eight normal volunteers in 3.2% sodium citrate (9:1 vol/vol). Baseline Sonoclot analysis (Sienco, Morrison, CO, USA) was performed with a recalculated blood sample, which was taken from a 1 ml-volume aliquot containing celite 1.5 mg. Analyses were repeated with a cuvette containing (final concentrations) heparin 1.5 U ml−1 plus either normal saline, AT 0.2 U ml−1 or tirofiban 70 ng ml−1. The addition of heparin and other drugs resulted in less than 1% dilution as compared to baseline samples. The following variables were measured: (i) clotting time (s); and (ii) clot rate (the gradient of the primary slope). Clotting time reflects the onset of fibrin formation, and clot rate reflects the rate of fibrin formation.

Blood samples were also obtained from patients undergoing elective cardiac surgery with concurrent heparin therapy (n=34). The durations of both of i.v. heparin therapy and concomitant drug administration were recorded. Patients with pre-existing haemostatic disorder, hepatic or renal disease, and concurrent anticoagulant therapy (GPIIb/IIIa antagonist, warfarin, or tissue plasminogen activator) were excluded. Baseline platelet count (×103 μl−1), prothrombin
time as the international normalized ratio (INR), and activated partial thromboplastin time (APTT, s) were recorded. Blood samples were obtained after induction of general anaesthesia but before full heparinization (400 U kg\(^{-1}\)). Blood (0.4 ml) was placed in kaolin-ACT cartridges (Medtronic, Parker, CO, USA) to determine baseline ACT, and into cartridges containing porcine intestinal heparin at final concentration of 1.5, 2.5, and 4.1 U ml\(^{-1}\). Blood was also added to cartridges that contained the same amount of heparin plus either AT (final concentration 0.2 U ml\(^{-1}\)), or tirofiban (final concentration 70 ng ml\(^{-1}\)), and ACTs were recorded. All tests were performed in duplicate. For determination of AT activity, blood samples from patients were collected into plastic tubes containing sodium citrate 3.2% (9:1 vol/vol), and centrifuged at room temperature for 15 min at 3000 g. Plasma was separated and stored at \(-80^\circ\)C until analysis. AT activity was determined using a commercially available kit (Coamatic Antithrombin; Chromogenix, Mondal, Sweden) with a coefficient of variation <8.0%.

**Statistical analysis**

All data are expressed as mean (SD). Differences between groups were analysed using analysis of variance with the Bonferroni correction. A \(P\) value \(\leq 0.05\) was considered significant.

**Results**

**Thrombin generation and platelet aggregation**

Addition of tirofiban (final concentration 0, 70, 140 and 280 ng ml\(^{-1}\)) to PRP increased lag time of thrombin generation in a concentration-dependent manner 9.67 (1.96), 11.77 (2.32), 12.57 (2.21) and 13.67 (1.94) min respectively, \(P<0.05\), but ETP was not affected (Fig.1). Heparin caused a concentration-dependent decrease in thrombin formation (ETP) and increase in lag time (Fig. 2). ADP did not induce thrombin generation in PPP. Tissue factor-induced thrombin activation in PRP was not effectively inhibited by tirofiban, but was completely inhibited by heparin (>0.5 U ml\(^{-1}\)) (data not shown). Tirofiban inhibited ADP-induced platelet aggregation in blood anticoagulated with 3.2% citrate or heparin (7 U ml\(^{-1}\)) (Fig. 3). Heparin per se did not significantly change the extent of ADP-induced platelet aggregation when compared with citrate anticoagulation.

**Whole blood clot formation**

**Sonoclot experiments**

Addition of tirofiban (70 ng ml\(^{-1}\)) or AT (0.2 U ml\(^{-1}\)) to heparinized blood samples (1.5 U ml\(^{-1}\)) caused significant prolongation of clot onset when compared with heparin only.
Additionally, there was a trend to a slower rate of clot formation on Sonoclot analyses in the tirofiban and AT groups, but the differences did not reach statistical significance when compared to heparin only (Table 1 and Fig. 4).

**ACT experiments**

Preoperative laboratory values were as follows: platelet count 194 (62) × 10^3 µL^-1, INR 1.04 (0.1), APTT 64.8 (24) s, and AT concentration 78.5 (23)%. Baseline ACT was 169 (33) s, and heparinization of the blood specimens to a final heparin concentration of 1.5, 2.5, and 4.1 U ml^-1 resulted in an increase in ACTs to 294 (105), 379 (118) and 583 (160) s respectively (Fig. 5). The addition of AT to heparinized blood samples further prolonged ACTs to 303 (66), 401 (95) and 673 (178) s respectively. However the difference was not significant when compared with heparin alone. When tirofiban was added to each target concentration of heparin, the corresponding increases in ACTs to 350 (57), 464 (87) and 802 (152) s were significantly larger at all heparin levels when compared with the heparin alone or heparin plus AT group (Fig. 5). When analyses of ACT changes were repeated with blood samples with AT activity <80% [mean 64.1 (12.3), n=21], similar results were obtained (data not shown).

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**Table 1** Sonoclot parameters. Mean (sd). *P<0.01 vs baseline; †P<0.01 vs heparin alone. AT = antithrombin; TIRO = tirofiban

<table>
<thead>
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<th></th>
<th>Baseline</th>
<th>Heparin only</th>
<th>Heparin+AT</th>
<th>Heparin+TIRO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Onset (s)</td>
<td>171 (26)</td>
<td>501 (113)*</td>
<td>652 (211)*</td>
<td>765 (150)*</td>
</tr>
<tr>
<td>Clot rate</td>
<td>22 (9.7)</td>
<td>7.3 (3.0)*</td>
<td>4.6 (2.3)*</td>
<td>1.3 (0.7)*</td>
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*(P<0.01). Additionally, there was a trend to a slower rate of clot formation on Sonoclot analyses in the tirofiban and AT groups, but the differences did not reach statistical significance when compared to heparin only (Table 1 and Fig. 4).*
In the present study, we hypothesized that tirofiban, a specific GPIIb/IIIa antagonist, would augment heparin in reducing thrombin generation and subsequent clot formation. We first measured thrombin generation in recalcified PRP, which was triggered by ADP or tissue thromboplastin (Innovin). Using the automated fluorescent assay system, the time course of thrombin generation and inactivation in platelet-rich or platelet-poor plasma can be quantified continuously using an artificial thrombin-specific, fluorogenic substrate and a thrombin calibrator of known constant activity. The graphic representation (thrombogram) of thrombin-catalysed fluorescence curves enabled us to characterize the effect of tirofiban on the lag time and the ETP. The ETP is different from other markers of thrombin activation (prothrombin fragment 1.2 or thrombin–antithrombin complex) because ETP describes the total capacity of plasma to generate thrombin, and the latter tests reflect the amount of thrombin that has already been generated. Compared with PT and PTT, which only detect prolonged lag time for bleeding tendency, the thrombin generation curve can be used to diagnose clinical coagulation disorders such as haemophilia and activated protein C resistance.

Tirofiban prolonged lag time when ADP was used to trigger thrombin formation. ADP-induced thrombin generation depends on platelets, because ADP does not induce thrombin in PPP. Although tirofiban did not reduce the total amount of thrombin generated (ETP), this does not exclude its potential anticoagulant effects because other clinically useful anticoagulants are known to cause a similar delay without reducing ETP. When tissue thromboplastin (Innovin) was used as a trigger, tirofiban did not effectively suppress thrombin generation. This can be explained by the direct activation of clotting factors seen in PPP. Our results are in agreement with Wegert and colleagues, who reported that abciximab inhibited platelet agonist-induced but not Innovin-induced thrombin generation. Heparin concentration-dependently delayed and reduced thrombin formation against ADP- or thromboplastin-induced thrombin generation in our assay system, but persistent thrombin generation and platelet activation seems to occur during CPB even when heparin concentrations and ACTs are adequately maintained.

Platelets are activated when only a small amount of thrombin is generated, and support prothrombinase (factor Va–factor Xa) complexes by providing a catalytic surface for thrombin generation. In the presence of heparin, free plasma thrombin and factor Xa are rapidly inhibited by AT in plasma. However, clot-bound thrombin is not inhibited by AT-heparin complex, and factor Xa also becomes resistant to inhibition when it is incorporated into the prothrombinase complex on the platelet surface. Additionally, factor IXa cannot be inhibited by AT when factor VIIIa and platelets are present.

Platelet aggregation is not inhibited with heparin anticoagulation per se (Fig. 3). Tirofiban inhibited ADP-induced platelet aggregation in citrate- and heparin-anticoagulated blood in a concentration-dependent manner. Moreover, platelet function seemed to be less inhibited with tirofiban at 35 ng ml⁻¹ in our study when heparin anticoagulation was
used (Fig. 3). This difference presumably resulted from the overestimation of platelet inhibition due to the calcium-chelating citrate.19

By inhibiting platelet aggregation, GPIIb/IIIa antagonists are effective in reducing propagation of procoagulant events. Reverter and colleagues showed that in vitro thrombin generation was delayed and reduced in the presence of abciximab.20 Tirofiban is a non-peptide tyrosine derivative that selectively and reversibly binds to platelet GPIIb/IIIa receptors. Plasma half-life of tirofiban is approximately 2 h; therefore, it is less likely to cause postoperative bleeding such as that seen in patients with abciximab therapy. In the second part of the study, we used clot-based methods because thrombin generation assay and platelet aggregation tests were not suitable for the evaluation of potentially additive effects of tirofiban and high-dose heparin. The in vitro tirofiban dose of 70 ng ml\(^{-1}\) was chosen on the basis of a rough approximation of administering a bolus dose of 5–10 \(\mu\)g kg\(^{-1}\) drug into a 70-kg adult.21

The first part of the clot-based study was performed with blood from healthy volunteers, using the Sonoclot analyser. The Sonoclot analyser detects clot gel formation as oscillatory changes, generating a continuous signal curve (signature), and subsequently parameters are calculated using proprietary computer algorithms. We used two calculated parameters of Sonoclot, clot formation time (onset) and clot rate. The former is comparable to activated clotting time, but the latter reflects the rate of fibrin polymerization, which is unique to Sonoclot. Both of these numerical parameters are calculated and then displayed on the instrument.

Sonoclot analyses showed that the onset of clot formation was further delayed with addition of AT or tirofiban in comparison with anticoagulation with heparin only (Table 1 and Fig. 4). The rate of subsequent clot formation also showed a trend of reduction with addition of tirofiban or AT. We expanded the experiments with a Medtronic–ACT system to study the effects of AT or tirofiban at varied heparin concentrations in blood samples from cardiac surgical patients treated before surgery with heparin. Although the Sonoclot analyser and the Medtronic–ACT system do not measure thrombin formation directly, the changes in blood viscosity that are detected with these monitors reflect thrombin-induced fibrin formation and platelet activation.22 23 In patient blood samples, tirofiban seemed to be more effective than AT in increasing ACT. Smaller increases in ACTs induced by addition of AT 0.2 U ml\(^{-1}\) may be related to the heparin concentrations used in this study (1.5–4.1 U ml\(^{-1}\)). In a previous study from our group, AT supplementation caused statistically significant ACT prolongations over non-AT-supplemented samples only at higher heparin concentrations (5.4–6.8 U ml\(^{-1}\)) in heparin-treated patients.24 Statistically significant ACT prolongations over non-supplemented samples were found with tirofiban at all heparin concentrations, suggesting that tirofiban prolongation of ACTs may be due to a mechanism other than heparin-AT mediated thrombin inhibition (Fig. 5).

Extensive platelet activation occurs during CPB, and platelets undergo morphological changes to form aggregates. Heparin anticoagulation is also susceptible to neutralization by platelet factor 4,25 which is released upon platelet activation. Addition of GPIIb/IIIa inhibitor to heparin has significantly improved the outcome of patients undergoing coronary interventions. Enhanced ACT responses to heparin in patients treated with abciximab, a classical GPIIb/IIIa inhibitor, have been reported.26 Prolongation of ACTs with abciximab could be attributed to other integrin receptors, such as \(\alpha_v\beta_3\) (vitronectin receptor), but our study shows that specific blockade of GPIIb/IIIa (\(\alpha_2\beta_3\)) receptors with tirofiban can effectively prolong ACTs. Platelet impairment induced by prostacyclin is also known to prolong ACTs up to 60%.27

Lack of platelet inhibition is one of the critical disadvantages of this current regimen of CPB anticoagulation. Strategies to supplement high-dose heparin with AT concentrate or recombinant AT may be effective, but the level at which AT activity should be supplemented to reduce thrombotic complications after CABG is not well defined.1 24 28 The mean value of AT activity in blood samples from cardiac patients was 78.5%. In the subgroup of patients who had AT activity <80%, the mean value was 64.1%, which is consistent with an AT concentration that is associated with clinical heparin resistance.1 5 Although tirofiban does not replete AT concentrations, this platelet inhibitor therapy may be useful in augmenting heparin anticoagulation when AT levels are moderately decreased. During CPB there is extensive activation of haemostatic and inflammatory systems, resulting in increased thrombin generation, which is only partially suppressed by the use of high doses of heparin.29 Suppression of platelets with tirofiban may potentially reduce thrombin formation on the activated platelet surface, and ultimately reduce platelet activation and clot formation on extracorporeal circuits. Preservation of platelet count and function by high-dose tirofiban has been reported in an experimental CPB in baboons.30 Experiences from the use of tirofiban immediately before7 or during CPB30 also provide evidence that tirofiban addition to heparin more effectively suppresses haemostatic activation on CPB.5 30 Bizzari and colleagues noted that the incidence of thrombocytopenia and haemostatic product use was less in 20 patients who received pre-operative tirofiban plus aspirin than in 68 patients who received only heparin plus aspirin.7

In summary, we have shown that a clinical dose of tirofiban can effectively delay platelet activation-mediated thrombin generation and prolong the ACTs of heparinized blood. Suppression of platelet function with tirofiban may be useful in preserving coagulatory function for cardiac surgical patients who present with clinical heparin resistance with moderately decreased AT concentrations.

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