Blood Coagulation, Fibrinolysis and Cellular Haemostasis

Blockade of GPIIb/IIIa by eptifibatide and tirofiban does not alter tissue factor induced thrombin generation in human endotoxemia

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Summary
Activated platelets facilitate thrombin generation by providing a catalytic surface on which coagulation activation occurs. The glycoprotein (GP) IIb/IIIa receptor might play a major role in this process as shown by in vitro and animal experiments. However, it is controversial whether the GPIIb/IIIa receptor facilitates tissue factor-induced thrombin generation in humans as well. We therefore investigated whether two clinically used GPIIb/IIIa antagonists (tirofiban and eptifibatide) may blunt TF-induced coagulation in humans.

Thirty male volunteers received 2 ng/kg endotoxin and standard doses of eptifibatide, tirofiban or placebo over 5 hours in a randomized, double-blind, placebo-controlled, double-dummy parallel-group trial. Markers of thrombin generation (prothrombin fragment 1+2, thrombin-antithrombin complexes), fibrinolysis (D-dimer, plasmin-antiplasmin complexes) as well as inflammatory markers (interleukin-6, tumor necrosis factor-α) were measured by enzyme linked immunoassays, TF-mRNA expression was quantified by RT-PCR. Neither eptifibatide nor tirofiban influenced LPS-induced coagulation activation or fibrinolytic activity. Additionally, the increase of TNF-α and IL-6 was similar in all groups.

In conclusion, GPIIb/IIIa blockade with eptifibatide or tirofiban did not influence TF-induced coagulation activation in human low grade endotoxemia.

Keywords
Eptifibatide, tirofiban, endotoxemia, randomized controlled trial, coagulation activation

Introduction
Results of several studies suggest that platelets play a key role in sepsis and its complications, like tissue factor (TF) triggered disseminated intravascular coagulation (DIC)(1). Activated platelets facilitate thrombin generation by providing a catalytic surface on which coagulation activation occurs (2). For example, tissue factor-induced thrombin generation correlated with platelet counts in an in vitro model using defibrinated plasma and gel-filtrated platelets (3).

The glycoprotein (GP) IIb/IIIa receptor might play a major role in this process. For two decades it has been known that fibrinogen, von Willebrand factor (vWF) and fibronectin are adhesive ligands for the GPIIb/IIIa receptor (4-8). Thereby, GPIIb/IIIa induces platelet aggregation. Yet, recent in vitro and animal experiments have shown that the GPIIb/IIIa complex binds prothrombin (9) and GPIIb/IIIa blockade with an antibody (c7E3 Fab, abciximab) inhibited TF-initiated thrombin generation in vitro (3, 10). Similarly, another monoclonal Ab (mAb; AZ-1) against GPIIb/IIIa decreased monocyte TF

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expression and consumption of coagulation factors in rabbits challenged with endotoxin (11). However, it is controversial whether the GPIIb/IIIa receptor facilitates TF-induced thrombin generation in humans as well (12-14).

Clinically used GPIIb/IIIa antagonists, like abciximab, tirofiban and eptifibatide, differ considerably in their pharmacodynamics: whereas the cyclic heptapeptide eptifibatide and the non-peptide tyrosine derivate tirofiban specifically bind to the GPIIb/IIa receptor, the monoclonal antibody abciximab also binds to the closely related αβ3 integrin and the β2 integrin CD 11b (15).

The infusion of endotoxin (lipopolysaccharide, LPS) into human volunteers is a standardized model to study systemic inflammation and TF-triggered systemic thrombin generation (16-19). Thus, we applied this model to investigate whether two selective GPIIb/IIIa antagonists (tirofiban and eptifibatide) may blunt TF-induced coagulation in humans.

Materials and methods

Study design

The trial was approved by the Ethics Committee of the University of Vienna and all participants gave written informed consent. We enrolled 30 healthy male volunteers (aged 18-39) in a double-blind, double-dummy (different infusion syringes), placebo-controlled trial, consisting of 3 parallel-groups (n = 10 per group). Basic examination included medical history, physical examination, laboratory parameters, and virological and drug screening. Additionally, study subjects were tested for hereditary thrombophilia.

Human endotoxemia is a well standardized model of systemic inflammation (18, 20) and detailed study procedures of the LPS model have been outlined in trials previously (20, 21). A bolus of 2 ng/kg National Reference Endotoxin (LPS, Escherichia coli; USP, Rockville, MD, USA) was infused in all subjects. In the current trial, volunteers received eptifibatide (Integrilin®), a kind gift of AESCA, Traiskirchen, Austria), tirofiban (Aggrastat®, MSD, Vienna, Austria) or 0.9% NaCl as placebo ten minutes later. GPIIb/IIIa inhibitors were given as standard licensed dosages used in everyday clinical practice for patients with acute coronary syndromes (ACS): eptifibatide (180 µg/kg bolus followed by 2 µg/kg/min for 5h), tirofiban (0.4 µg/kg/min for 30’ followed by 0.1 µg/kg/min for 4.5 h). Because of safety concerns, we limited the infusion period to 5h and did not test abciximab which has a long half-life and may cause severe thrombocytopenia in up to 2% of treated subjects, even in healthy volunteers (22, 23).

Blood sampling and analyses

Blood samples were collected into citrated or EDTA anticoagulated evacuated-tubes (Vacutainer, Becton Dickinson, Austria) by venipuncture half an hour before and 1, 2, 3, 4, 5, 6 and 24 hours after LPS-infusion. Plasma was obtained by centrifugation at 3000g (15 min at 4° C) and stored in 0.5 ml aliquots at −80° C until batch analysis.

All coagulation and inflammatory parameters were measured by enzyme immunoassays: plasma levels of prothrombin fragment (F1+2, Behring, Marburg, FRG) (24), D-dimer (Boehringer Mannheim, FRG), thrombin-antithrombin III complexes (TAT, Enzygnost TAT micro, Behring), plasmin-antiplasmin complexes (PAP, Enzygnost PAP micro, Behring), tumor necrosis factor-α and interleukin-6 (high sensitivity TNF-α and IL-6, R&D Systems, MN, USA) (20, 25).

Blood counts were performed with a cell counter (Sysmex, Milton Keynes, UK).

Monocyte counts were calculated from scatter histograms obtained by a flow-cytometer (Becton Dickinson, Vienna, Austria) at 0h, 2h, 5h and 24h.

Real-time quantitative PCR (RT-PCR)

All blood samples were immediately processed to avoid storage induced alterations in mRNA levels (26). After preparing total RNA with the QuiAmp RNA easy kit (Quiagene, Valencia, CA, USA), mRNA was directly transcribed into cDNA using the RT-Reagent kit (Applied Biosystems, Foster City, CA, USA) and stored at −80° C until analysis.

For TF-mRNA quantification the Abi Prism 7700 (Applied Biosystems) was used. Primers were designed by Primer Express Software (Applied Biosystems) and synthesized based on the human TF cDNA sequence: 680 F 5’-CCCGAACAGTTAACC GGAA AGA-3’ and 773R 5’-GCTCCA ATGTGTA-GAATTTCTCTGA-3’, TaqMan probe 711T FAM CTC- CTGGCCCATACACTCTACC GG TAMRA. 18s was used as a housekeeping gene for multiplexing (Applied Biosystems) because of its stable expression under endotoxemia (own unpublished data). TF-mRNA was normalized against the reference gene (18s) according the CT method (26) and data is expressed as fold-increase over baseline values. Dilution curves of TF-mRNA obtained from LPS-incubated blood samples revealed linearity (r = 0.999) of the assay up to 37.5 cycles, which was set as the limit of sensitivity (and also as a baseline for conservative statistical calculations). In our previous trials ((27 and data from an unpublished trial), maximal TF-mRNA up-regulation was observed after 5h. Thus, TF-mRNA determination was restricted to basal and peak levels (5h).

The Rapid Platelet Function Analyzer (RPFA; Ultegra method)

In the Ulategra-test, platelet activation by a thrombin receptor-activating peptide (TRAP) induces GPIIb/IIIa receptor dependent agglutination of fibrinogen-coated beads. Changes in light transmission are quantified as platelet activation units (PAU). The relative decrease in PAU-values closely reflects GPIIb/IIIa receptor blockade (28).
**Data analysis**

Data are expressed as mean and 95% CI or median and interquartile ranges (IQR) in case of skewed distribution. All statistical comparisons of continuous variables were made with non-parametric tests. The Kruskal Wallis ANOVA was used to test differences between groups. The Friedman ANOVA and the Wilcoxon test for post hoc comparisons were used to assess time dependent changes in outcome variables within groups. A two tailed p-value of < 0.05 was considered significant.

All statistical calculations were performed using a commercially available statistical software (Statistica Vers. 5.0, Tulsa, OK). Power calculation was done as previously described (29).

**Results**

Baseline characteristics of 30 healthy male volunteers are outlined in table 1. There was no significant difference in any parameter between treatment groups.

**Prothrombin fragment (F1+2), TAT, D-dimer and PAP**

Four hours after LPS-infusion, F1+2 levels increased approximately 7-fold in all groups (p <0.001 vs. baseline, p = n.s. between groups, Fig. 1). Similarly, TAT-levels increased 11 to 14-fold in all groups (p = n.s. between groups, Fig. 1).

D-dimer plasma levels rose to a maximum of 0.33 μg/mL (0.19-0.48) in the placebo group, to 0.33 μg/mL (0.20-0.46) in the eptifibatide group and to 0.39 μg/mL (0.16-0.61) in the tirofiban group with slight differences between time courses (p = 0.005 vs. baseline for all groups, p = n.s. between treatments, Fig. 1).

LPS-infusion induced early fibrinolytic activity as reflected by a two-fold increase of PAP levels in the placebo group after 3 h. Similarly, we observed a two-fold increase in PAP-levels after 3 h in the tirofiban and after 2 h in the eptifibatide group (p = n.s. between groups, data not shown).

**TF-mRNA expression**

Basal TF expression (<37.5 cycles) could be detected in 24/30 study subjects.

In all groups, TF mRNA increased by a maximum of 8-10-fold 5h after LPS-infusion (p = n.s. between groups, data not shown).

**IL-6, CRP and TNF-α**

After 2h IL-6 levels rose to a maximum of 70 pg/mL (IQR 40-124) in the placebo group, to 37 pg/mL (IQR 14-46) in the tirofiban group and to 69 pg/mL (IQR 14-77) in the eptifibatide group (p <0.001 vs baseline in all groups, p = n.s. between treatments, Fig. 2). TNF-α levels increased on average 260-fold (225-282) at 3h with no significant difference between treatment groups (Fig. 2). CRP-levels averaged 1.7 mg/dL (1.4-2.0) at 24h (p = n.s. between groups).

**Rapid Platelet Function Assay (RPFA)**

PAU-values were measured in order to document clinically relevant GPIIb/IIIa receptor blockade. PAU-values decreased by 88% (IQR 87-93%) after tirofiban bolus infusion and by 96% (IQR 91-100%) after eptifibatide bolus. During continuous infusion of tirofiban and eptifibatide PAU-values decreased by 80% (IQR 72-88%) and 92% (IQR 87-100%), respectively (data not shown).

**Table 1:** Baseline characteristics of healthy volunteers.

<table>
<thead>
<tr>
<th></th>
<th>Placebo (n=10)</th>
<th>Tirofiban (n=10)</th>
<th>Eptifibatide (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>27 ± 5</td>
<td>25 ± 2</td>
<td>26 ± 3</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>23.0 ± 2.9</td>
<td>23.3 ± 2.5</td>
<td>23.1 ± 2.5</td>
</tr>
<tr>
<td>Neutrophil counts, 10⁹/L</td>
<td>2.4 ± 0.8</td>
<td>2.7 ± 0.8</td>
<td>2.5 ± 0.7</td>
</tr>
<tr>
<td>Lymphocyte counts, 10⁹/L</td>
<td>2.4 ± 0.6</td>
<td>2.1 ± 0.2</td>
<td>1.9 ± 0.3</td>
</tr>
<tr>
<td>Monocyte counts, 10⁹/L</td>
<td>0.3 (0.2-0.4)</td>
<td>0.4 (0.3-0.4)</td>
<td>0.3 (0.2-0.4)</td>
</tr>
<tr>
<td>Platelet counts, 10⁹/L</td>
<td>210 ± 24</td>
<td>230 ± 62</td>
<td>229 ± 60</td>
</tr>
<tr>
<td>F₅/₇₇, nmoL/L</td>
<td>0.4 ± 0.07</td>
<td>0.4 ± 0.12</td>
<td>0.4 ± 0.14</td>
</tr>
<tr>
<td>TAT⁺, μg/L</td>
<td>2.2 (&lt;1.9-2.6)</td>
<td>1.9 (&lt;1.9-2.0)</td>
<td>&lt;1.9</td>
</tr>
<tr>
<td>D-dimer, μg/mL</td>
<td>0.18 ± 0.09</td>
<td>0.25 ± 0.20</td>
<td>0.17 ± 0.10</td>
</tr>
<tr>
<td>PAP, μg/L</td>
<td>206 ± 101</td>
<td>205 ± 83</td>
<td>241 ± 122</td>
</tr>
<tr>
<td>TF-mRNA expression*, cycles</td>
<td>36 (35-37)</td>
<td>36 (35-37)</td>
<td>36 (36-37.5)</td>
</tr>
<tr>
<td>IL-6, pg/mL</td>
<td>1.3 (1.2-2.2)</td>
<td>1.6 (1.3-3.7)</td>
<td>1.3 (1.1-1.9)</td>
</tr>
<tr>
<td>TNF-α, pg/mL</td>
<td>1.3 ± 0.48</td>
<td>1.0 ± 0.32</td>
<td>1.0 ± 0.19</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SD or median and the interquartile ranges (IQR) in case of highly skewed distribution.
Blood cell counts

As expected (20), neutrophil counts dropped from average 2.5 (2.2-2.8 \times 10^9/L) 90 min after LPS-infusion [minimum 1.8 (1.25 \times 10^9/L) for the placebo, 2.0 (1.5-2.6 \times 10^9/L) for the tirofiban and 1.9 (1.3-2.6 \times 10^9/L) for the eptifibatide group] and recovered within 30 min (p = n.s. between groups). In all groups, lymphocyte counts declined steadily to 25-30% of the basal values over 5h time (p = n.s. between treatments). Monocyte counts markedly declined (88-95%) after 2 h [median 0.02 (IQR 0.01-0.05) in the placebo, 0.04 (IQR 0.04-0.05) in the tirofiban and 0.03 (IQR 0.02-0.05) in the eptifibatide group] and recovered after 5h. There was no significant difference in magnitude or course in monocyte counts between treatment groups (p = n.s, data not shown).

LPS-infusion decreased platelet counts by 15%-20% at 4 h in all groups without significant difference, but importantly, GPIIb/IIIa antagonists did not induce thrombocytopenia.

Discussion

Controversial data exist about the potential inhibitory effects of GPIIb/IIIa receptor antagonists on thrombin generation, as described in detail below (12-14). We therefore evaluated the effect of two specific GPIIb/IIIa inhibitors in an in vivo model of systemic TF-induced coagulation without concomitant anticoagulants or antiplatelet drugs.
Our results demonstrate that neither GPIIb/IIIa antagonist influenced the TF-induced pathway of coagulation as measured by TF-mRNA expression and thrombin generation quantified by F1+2 and TAT-levels or fibrinolytic activity (D-dimer, PAP). While F1+2 responses to LPS were somewhat less pronounced than in earlier trials (16, 30), the magnitude of F1+2 responses fall in the range of some of our more recent trials (18, 27). As compared to placebo, we had an 80% power to detect a 35-50% lower F1+2 formation in the active treatment groups.

In vitro studies
Abciximab inhibited TF-triggered thrombin generation by about 50% (3) in an in vitro model using defibrinated plasma and gel-filtered platelets. As abciximab is not specific for the GPIIb/IIIa receptor, inhibition of thrombin formation may have been due to blockade of the αβ3 vitronectin receptor (31-33). This is supported by the finding that abciximab inhibited thrombin generation more effectively than a specific anti-GPIIb/IIIa mAb in this model (3).

Interestingly, in a model where hemostasis is initiated with recombinant human TF in minimally modified whole blood, neither abciximab nor eptifibatide affected thrombin generation profiles at high TF-concentrations (200 pM), whereas both drugs inhibited thrombin generation at low TF-concentrations (25 pM) by 50-75% (34). Along this line, abciximab inhibited thrombin generation by washed platelets, particularly when incubated with low levels of TF (35). Tirofiban, eptifibatide and abciximab also inhibited thrombin formation in another in vitro study (36).

In contrast, abciximab did not affect thrombin generation under high shear conditions in vitro, whereas a mAb against von Willebrand factor effectively reduced thrombin generation (~70%) by washed human platelets (37).

Animal studies
In rabbits challenged with endotoxin monocyte TF expression was reduced by an anti- GPIIb/IIIa mAb (~70% after 5 days) (11). Similarly, abciximab attenuated coagulation activation by 60-70% and protected against renal insufficiency in baboons treated with a sublethal E. coli dose and C4b-binding protein (38). This may at first sight seem to contradict our results. However, abciximab’s crossreactivity with the αβ3 and the αMβ2 integrins is expected to block several other mediators of coagulation and inflammation, including the complement factor iC3b, fibrinogen, the coagulation factor FX or high molecular weight (HMW) kininogen (33), whereas GPIIb/IIIa selective antagonists may not have such effects.

On the other hand, in animals challenged with sublethal doses of endotoxin (11), GPIIb/IIIa antagonists may exert their beneficial effect by inhibition of platelet aggregation in the microcirculation.

Finally, continuous infusion of low or high dose tirofiban decreased thrombin generation in baboons undergoing cardiopulmonary bypass, which is mainly a model for contact activation rather than TF-induced coagulation (39). While contact activation is not considered a physiological trigger of coagulation in vivo, this study could point to an interesting mechanism of action, which could be exploited during extracorporeal circulation.

Patients studies
Importantly, our results are in line with recently published data showing that abciximab did not affect thrombin generation (F1+2 levels) in acute coronary syndrome (ACS) patients without subsequent percutaneous coronary intervention (PCI)(12): In such patients, it is conceivable that increased thrombin generation occurs at sites of arterial stenosis and thereby at high shear rates, where abciximab proved inefficacious in vitro (37).

Similarly, treatment with abciximab only led to significant reduction in F1+2 levels in a post-hoc comparison of before/after treatment differences in patients undergoing PCI: However, comparing absolute F1+2 values, no significant difference was found whereas abciximab markedly prolonged the activated clotting time (ACT). That latter test system strongly depends on platelets and thus on GPIIb/IIIa antagonists (13, 40).

Besides, patients in both trials concomitantly received heparin, whose anticoagulant action may be enhanced by GPIIb/IIIa antagonists (41).

Thus, a total of three clinical trials (including this one) provide evidence against a relevant contribution of the GPIIb/IIIa complex in TF-mediated thrombin formation.

Our study revealed that neither eptifibatide nor tirofiban influenced the release of TNF-α and IL-6. Consistently, levels of PAP and CRP, which are regulated by TNF-α and IL-6, respectively in this model (42), were not different between treatment groups. None of the previous animal studies reported on inflammation markers (11, 38, 39). However, in patients undergoing PCI abciximab prevented a further 2-fold increase of IL-6 levels with only a marginal effect on CRP-values (33). Alternatively, the LPS-stimulus, inducing a relatively strong increase in IL-6-levels, may have masked minor effects on IL-6. However, levels of CRP in our trial at 24h are comparable to those of the patient trial (43).

Finally, we have to state that our study has at least one evident limitation: our model produces a relatively mild LPS induction of TF, platelet activation and relatively few leukocyte-platelet aggregates (20, 44-47). In contrast, in septic shock one would expect a strong LPS induction of TF-expression and more leukocyte-platelet aggregates. Thus, a platelet inhibitor...
could be expected to have a greater impact on thrombin generation in septic shock than in low grade endotoxemia.

In conclusion, neither epibatidine nor tirofiban inhibited TF-induced coagulation activation in human low grade endotoxemia. Neither drug therefore appears to be a suitable candidate for therapeutic intervention in systemic inflammation induced DIC. Thus, in clinical practice tirofiban and epibatidine seem to primarily act as anti-platelet agents rather than by interference with tissue factor driven thrombin generation.

Acknowledgments
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