Comparative efficacy of fibrinogen and platelet supplementation on the in vitro reversibility of competitive glycoprotein IIb/IIIa (αIIb/β3) receptor-directed platelet inhibition

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Background  Platelet surface glycoprotein (GP) IIb/IIIa (αIIb/β3) receptor inhibition, by preventing fibrinogen binding and platelet aggregation, concomitantly attenuates arterial thrombotic capacity and impairs protective hemostasis, 2 divergent platelet-dependent processes. Because the currently available Food and Drug Administration–approved small molecule GP IIb/IIIa receptor antagonists are considered “competitive” inhibitors and because there is limited information on the reversibility of platelet inhibition by fibrinogen or platelet supplementation, the following series of in vitro experiments were performed.

Methods and Results  Washed platelets from 24 healthy volunteers were suspended in Tyrodes buffer and incubated with achievable (in vivo) steady-state concentrations of either tirofiban or eptifibatide before activation with TRAP (thrombin receptor agonist peptide) (15 μmol/L). Platelet aggregation was inhibited by 40% to 50%, but reversal was achieved by fibrinogen supplementation in a concentration-dependent manner. In a separate series of in vitro experiments, platelet inhibition exceeding 90% was established with tirofiban (average concentration 9.28 μg/L) and eptifibatide (average concentration 95.4 μg/L). Recovery of platelet aggregation to at least 50% was achieved after the addition of fibrinogen (0.76-0.80 g/L), platelets (2.4 × 10¹¹/L), or their combination. There was an inverse relationship between plasma baseline fibrinogen and the amount of supplemental fibrinogen required to restore platelet aggregability (r = -0.60, P < .01).

Conclusions  The reversibility of GP IIb/IIIa–directed platelet inhibition is influenced by cell surface receptor availability and the intrinsic pharmacodynamic mechanism of action. Fibrinogen supplementation with fresh frozen plasma or cryoprecipitate either alone or in combination with platelet transfusion, represents an important and readily available treatment consideration for restoring hemostatic potential and managing major hemorrhagic complications associated with the administration of small molecule competitive GP IIb/IIIa receptor antagonists. (Am Heart J 2001;142:204-10.)
vitro study was performed to determine the comparative ability of fibrinogen and platelet supplementation to restore platelet aggregability in the presence of small molecule GP IIb/IIIa receptor–directed inhibitors.

**Material and methods**

A total of 24 healthy blood donors (average age 35 years, range 25 to 52 years) were recruited for the study and advised according to a protocol approved by the Committee for Human Subjects Participating in Research, University of Massachusetts Medical School. All donors were screened carefully to exclude those with either a family or personal history of thrombosis (thrombophilia), bleeding (coagulopathy), or medication use within the prior 2 weeks that could have influenced platelet aggregability (aspirin, nonsteroidal anti-inflammatory agents).

**Measurement of platelet aggregation**

All blood samples were obtained by an experienced phlebotomist using a 21-gauge butterfly needle. Washed platelets (2 × 10^11/mL) suspended in Tyrodes buffer were incubated with achievable concentrations (in vivo) of tirofiban and eptifibatide before activation with thrombin receptor agonist peptide (TRAP) (15 μmol/L). Inhibition of platelet aggregation was determined by standard turbidimetric platelet aggregometry after the addition of fibrinogen in concentrations ranging from 0.5 to 2.0 g/L.

In a second series of experiments, 9 parts blood was mixed with 1 part diluted heparin with a final concentration at 10 IU/mL. Platelet-rich plasma (PRP) was obtained by centrifuging blood at 175g for 15 minutes at 24°C. Platelet concentrations in whole blood and PRP were determined by a Coulter counter. The heparin-anticoagulated blood was diluted with saline solution at 1:1 and then transferred to individual cuvettes. The diluted blood was preincubated at 37°C for 5 minutes before platelet aggregation measurements were performed. After activation with adenosine diphosphate (ADP) (3 μmol/L), the electrical impedance change was recorded for 6 minutes. For each individual, baseline electrical impedance was recorded followed by the determination of GP IIb/IIIa concentration required to inhibit platelet aggregation by 90%. The sample was then incubated in PRP with either tirofiban or eptifibatide for 5 minutes at 37°C, followed by the addition of saline solution, ascending concentrations of fibrinogen, or platelets at 2 concentrations (1.2 × 10^11/L and 2.4 × 10^11/L). After an additional 5 minutes of incubation, ADP was added and electrical impedance change was recorded for 6 minutes. All samples were run in triplicate.

The whole blood aggregometer (model 560-ca) used to measure platelet aggregation by impedance was a product of Chrono-Log (Havertown, Pa). Tirofiban and eptifibatide were provided by Merck Pharmaceuticals (Rahway, NJ) and COR Therapeutics (South San Francisco, Calif), respectively. Human fibrinogen was obtained from Sigma (St Louis, Mo) and human α-thrombin from Calbiochem (La Jolla, Calif). All chemicals were of reagent grade.

**Fibrinogen quantification.** Blood samples for fibrinogen measurement were collected with a one tenth volume of 3.8% sodium citrate as an anticoagulant. Platelet-poor plasma (PPP) was separated by centrifuging blood at 2800g for 15 minutes at 24°C and then rapidly frozen in aliquots at -80°C until analyzed. A modified thrombin time was used to quantify the plasma concentration of fibrinogen. Briefly, the plasma to be tested was diluted 1:10 or 1:20 with 50 mmol/L tris-(hydroxymethyl)-aminomethane (TRIS)-hydrochloric acid buffer with 2 mmol/L sodium chloride and 2 mmol/L calcium chloride, pH 7.4. Human α-thrombin reagent was prepared at 100 IU/mL in the same buffer. Before the plasma was tested, a standard curve was prepared. Human fibrinogen was reconstituted with distilled water. Four dilutions were prepared with fibrinogen concentrations of 0.6, 1.6, 3.2, and 4.8 mg/mL. For each dilution, 0.2 mL was transferred to a reaction tube, warmed to 37°C, and tested by adding 0.1 mL of thrombin reagent. Time from addition of thrombin to clot formation was recorded and plotted on a graph against concentration. In the same way, sample plasma was tested for thrombin time, and time was transferred to fibrinogen concentration according to the standard curve.

**Statistical analysis.** Data were expressed as the mean ± SD, unless otherwise indicated. The Student t test and linear regression were used for statistical analyses. Values were considered statistically significant when P < .05. The computer program SPSS for Windows (SPSS, Chicago, Ill) was used.

**Results**

Platelets obtained from healthy donors were inhibited by 40% to 50% (in response to TRAP) with intermediate concentrations of tirofiban (42 nmol/L) and eptifibatide (333 nmol/L). Fibrinogen supplementation in concentrations of 0.5, 1.0, and 2.0 g/L restored platelet aggregability to 80.0%, 80.0%, and 82%, respectively, with tirofiban and 80.0%, 84.0%, and 84%, respectively, with eptifibatide (Figure 1).

Platelet inhibition in whole blood, as determined by impedance aggregometry, after incubation with saline solution control or an intermediate concentration of tirofiban either alone or in combination with an increasing concentration of fibrinogen is summarized in Figure 2. A. Recovery of platelet aggregability (in response to ADP stimulation) after the addition of fibrinogen is summarized in Figure 2. B. A ≥50% recovery was achieved in 16 of 24 (67%) individuals with, on average, 0.8 g/L of supplemental fibrinogen. There was an inverse relationship between the baseline plasma fibrinogen concentration and the amount of supplemental fibrinogen required to achieve ≥50% platelet aggregation in the presence of tirofiban (r = -0.60, P < .01).

Platelet inhibition achieved with eptifibatide and its reversal with supplemental fibrinogen is depicted in Figure 3. A. A recovery in platelet aggregation to ≥50% was achieved in 16 of 24 (67%) individuals who, on average, 0.76 g/L supplemental fibrinogen (Figure 3, B). As with tirofiban, there was an inverse relationship between the baseline plasma fibrinogen concentration and the amount of supplemental fibrinogen required to achieve ≥50% platelet aggregation in the presence of eptifibatide (r = -0.60, P < .01).
Recovery of platelet aggregation with platelet supplementation

Platelet inhibition of >90% in response to ADP (3 µmol/L) was achieved with tirofiban (average concentration 9.28 ± 1.78 µg/L). Recovery of platelet aggregation was determined after supplementation with increasing concentrations of single donor platelets. Average recovery was 25.8% ± 13.9%, 54.6% ± 21.9%, 74.8% ± 25.9%, and 90.0% ± 27.2% in response to 1.2 × 10¹¹/L, 2.4 × 10¹¹/L, 3.6 × 10¹¹/L, and 4.8 × 10¹¹/L platelet supplementation, respectively. A separate series of in vitro experiments carried out with eptifibatide (average concentration to achieve >90% platelet inhibition 95.4 ± 18.3 µg/L) revealed recovery rates of 26.7% ± 13.6%, 53.1% ± 18.9%, 70.8% ± 22.9%, and 87.8% ± 25.1%, respectively.

Recovery of platelet aggregation with fibrinogen and platelet supplementation

Platelet inhibition (>90% in response to ADP [3 µmol/L]) was achieved with tirofiban in varying concentrations (average 9.28 ± 1.78 µg/L). Recovery of platelet aggregation was determined after supplementation with either fibrinogen alone (0.75 g/L), platelets alone (1.2 × 10¹¹/L, 2.4 × 10¹¹/L), or their combination. The addition of platelets was associated with a 30% to 54% recovery of aggregability, whereas the combination of fibrinogen and platelets restored aggregation response by 40% to 67%. Similar observations were made with eptifibatide (Tables I and II).

Discussion

Platelet surface GP IIb/IIIa receptor inhibition, by preventing fibrinogen binding and platelet aggregation in response to a wide complement of biochemical and mechanical agonists, concomitantly attenuates arterial thrombotic capacity and hemostatic potential. Our series of in vitro studies suggests that blood products rich in fibrinogen represent an important treatment consideration for serious hemorrhagic complications associated with the administration of small molecule competitive GP IIb/IIIa receptor antagonists. They also suggest that platelet transfusions may play a role in drug (effect) reversibility.

GP IIb/IIIa receptor antagonist reversibility

The GP IIb/IIIa receptor antagonists, although collectively included within a specific class of platelet inhibitors, differ in molecular size, biology (on and off rates from the receptor), binding specificity/selectivity, plasma half-lives, and clearance; each of these factors influence an agent’s reversibility profile. Abciximab is a large molecule (50,000 d) that has a short plasma half-life (10 minutes) but remains biologically active on the platelet surface for 12 to 24 hours. In contrast, the small molecule antagonists tirofiban and eptifibatide have molecular weights of 800 and 500 d, with plasma and biologic half-lives of 90 to 120 minutes and 2 to 4
hours, respectively. Abciximab undergoes resorption and proteolytic breakdown within the kidney, whereas tirofiban is excreted via renal and hepatic routes. Eptifibatide is excreted primarily through renal mechanisms.

The affinity of a drug for its receptor is typically represented by the dissociation constant (K_D). At equilibrium, K_D is equal to the product of the unbound drug concentration (in plasma) and the unbound receptor concentration divided by the drug-receptor (bound) complex concentration. Accordingly, drugs that have a high K_D (low affinity) have a large unbound (free) concentration at steady state, whereas those with a low K_D (high affinity) are predominantly in a receptor-bound state, with a proportionally low concentration of unbound drug within the circulation. These fundamental principles are important when considering the reversibility of platelet inhibition achieved with current intravenous GP IIb/IIIa receptor antagonists.

The monoclonal antibody abciximab is a relatively high affinity GP IIb/IIIa antagonist with a K_D of 5 mmol/L. After an intravenous bolus, a large proportion of the drug is rapidly bound to platelet GP IIb/IIIa receptors—the unbound pool is rapidly cleared from the circulation. In contrast, the small molecule antagonists tirofiban and eptifibatide, with K_D values of 15 mmol/L and 120 mmol/L, respectively, have large unbound pools of drug within the circulation.

Reversibility with fibrinogen supplementation

Binding of soluble fibrinogen to the “ligand-receptive” GP IIb/IIIa receptor is essential for platelet aggregation and arterial thrombosis. Electron microscopy has shown that the globular head of the receptor interacts with the distal end of the fibrinogen molecule at several distinct sites (two RGD sequences in each of the α chains and a dodecapeptide sequence at the carboxy-terminus of each γ chain). Under normal circumstances, stimulated platelets can bind 40,000 or more fibrinogen molecules. Recruitment of receptors from “internal storage pools” may allow another 20,000 molecules to bind the platelet surface. Because plasma fibrinogen concentrations exceed the receptors dissociation constant by 30-fold, binding sites are rapidly saturated and removal of the molecule from its receptor is difficult, a biochemical event known as “time-dependent stabilization.”

Fundamentally, the GP IIb/IIIa receptor antagonists prevent platelet aggregation by preventing fibrinogen...
binding. For the small molecule antagonists, this is achieved by direct competition for fibrinogen-binding sites on the GP IIb/IIIa complex. In our series of in vitro experiments, fibrinogen supplementation restored platelet aggregability in the presence of tirofiban and eptifibatide. The effect was concentration dependent, achieved with an average concentration of 0.8 g/L, and inversely related to the baseline plasma fibrinogen level. Thus in patients with ACS and a normal or modestly elevated plasma fibrinogen level,25 approximately 2400 mg of supplemental fibrinogen (8 units of fresh frozen plasma) would be required at peak tirofiban/eptifibatide plasma concentration to restore platelet aggregation to at least 50% of normal (150 mg of supplemental fibrinogen increases plasma concentration by 5 mg/dL). In patients with normal renal function (and rapid clearance of tirofiban/eptifibatide) the amount of supplemental fibrinogen required to restore platelet aggregation would be less, particularly if the drug infusion had been terminated for 1 hour or more. Approximately one half the calculated dose would be needed beyond 4 hours (from infusion cessation) with even smaller requirements up to 12 hours. Similarly, a lower replacement volume would be required to restore platelet aggregability in patients with a baseline fibrinogen above 3.2 g/L.

Although our study did not examine specific mechanisms of reversibility, we hypothesize that exogenous fibrinogen competes with tirofiban and eptifibatide for platelet surface-binding sites. Thus, with GP IIb/IIIa antagonists characterized by relatively low receptor affinity, rapid “off” rates and high unbound (free) plasma concentrations, fibrinogen substrate is a readily available means to effectively restore platelet-mediated hemostatic potential.

Reversibility with platelet supplementation

Inhibition of platelet aggregation by the GP IIb/IIIa antagonists requires drug binding to a large proportion of surface receptors (>70%), supporting a “threshold” for occupancy below which an inhibitory effect is markedly diminished (or not achieved).26 Because plasma concentrations of unbound drug are relatively high for both tirofiban and eptifibatide (compared with abciximab), it has been assumed that platelet transfusions would not effectively reverse their inhibitory effects.11 Our findings suggest that this assumption may not be entirely correct. A restoration of platelet aggregation to 50% of normal was achieved in the presence of steady-state drug concentrations by increasing the platelet count by $2.4 \times 10^{11}$/L. Although further investigation is needed, the restoration of platelet aggregability suggests either that transfused platelets are not “ligand receptive” (ie, less likely to bind tirofiban or eptifibatide), the proportion of bound receptors is below threshold for platelet inhibition (absolute receptor occupancy), or the extent of molecule binding per receptor is inadequate to prevent fibrinogen recognition (relative receptor occupancy).

The combined strategy of providing fibrinogen substrate to competitively block unbound GP IIb/IIIa receptors and platelets to “dilute” plasma pools (of inhibited platelets) had the greatest impact on platelet responsiveness and aggregation.

Study limitations

Our in vitro experiments used concentrations of tirofiban and eptifibatide commonly achieved (with currently recommended dosing strategies) in plasma at steady state. Higher plasma concentrations, such as those reached immediately after an intravenous bolus or in patients with reduced clearance (renal insufficiency), would influence the amount of fibrinogen or platelet supplementation required for reversal. Although it seems likely that the reversibility attributable to fibrinogen is best explained by competition (with tirofiban or eptifibatide) for available GP IIb/IIIa surface receptors, we cannot exclude the possibility of fibrinogen-mediated platelet activation (with expression of additional GP IIb/IIIa receptors) or fibrinogen-

### Table II. Effect of fibrinogen and platelet supplementation on the recovery of platelet aggregation in the presence of eptifibatide

<table>
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<tr>
<th>Treatment</th>
<th>Fibrinogen (0.75 g/L) (A)</th>
<th>Platelet 1 (1.2 $\times 10^{11}$/L) (B)</th>
<th>Fibrinogen + platelet 1 (C)</th>
<th>Platelet 2 (2.4 $\times 10^{11}$/L) (D)</th>
<th>Fibrinogen + platelet 2 (E)</th>
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Table II. Effect of fibrinogen and platelet supplementation on the recovery of platelet aggregation in the presence of eptifibatide.
Summary/clinical implications

The standard of care for patients with acute coronary syndromes at high risk for myocardial infarction and cardiac death includes an intravenous GP IIb/IIIa receptor antagonist. Although the cost-effectiveness and safety profiles for these drugs have been established, situations do arise where reversibility of their platelet inhibiting properties (and prompt restoration of hemostatic capacity) is critical. Our in vitro experiments support the use of fibrinogen supplementation (cryoprecipitate, fresh frozen plasma) for patients with moderate bleeding and combined platelet and fibrinogen supplementation for those with severe/life-threatening hemorrhagic complications (or moderate bleeding accompanied by thrombocytopenia) associated with the administration of small peptide/nonpeptide competitive GP IIb/IIIa receptor antagonists. The decision to “reverse” an antithrombotic agent(s) must be based on a comprehensive evaluation of potential risks and benefits and, under ideal circumstances, documented through rapid and reliable point-of-care laboratory measurement tools.

References


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