Platelet Activation With Unfractionated Heparin at Therapeutic Concentrations and Comparisons With a Low-Molecular-Weight Heparin and With a Direct Thrombin Inhibitor

Zihui Xiao, MSc; Pierre Théroux, MD

Background—The growing use of heparin in acute thrombotic disorders, coupled with the availability of many new antithrombotic agents, emphasizes the need for adequate characterization of the platelet effects of the various anticoagulants. Controversial platelet effects have been reported with heparin (eg, enhanced platelet activation in vitro with high doses and no such effect in vivo at therapeutic doses). This study examined platelet receptor activation and platelet aggregation at therapeutic concentrations of unfractionated heparin (UFH), of enoxaparin, a low-molecular-weight heparin, and of argatroban, a direct thrombin inhibitor.

Methods and Results—Platelet P-selectin (CD62) and activated GP IIb/IIIa (PAC-1) expression on platelet membrane was quantified in whole blood as well as platelet aggregation in platelet-rich plasma in 43 patients with unstable angina before and during treatment with UFH or enoxaparin. Studies were also carried out in blood of seven normal volunteers after addition ex vivo of UFH (0.25 U/mL), enoxaparin (0.25 U/mL), argatroban (1 ng/mL), and normal saline. UFH in patients with unstable angina increased the percentage of circulating platelets positive to PAC-1 from 2.7±1.7% to 4.4±3.4% (P<.05) and to CD62 from 1.6±0.9% to 2.7±1.5% (P<.01). Platelets were also hyperresponsive to stimulation with ADP and with the thrombin-receptor agonist peptide. Aggregation to ADP increased from 6.8±4.6% to 11.2±7.0% and to TRAP from 5.2±3.5% to 11.1±6.0% (P<.001). The addition of UFH to blood of normal volunteers resulted also in activation of GP IIb/IIIa receptors, expression of P-selectin, and enhanced platelet aggregation. Enoxaparin had only minor effects on platelet activation in vivo and ex vivo, and argatroban, evaluated ex vivo, had no detectable effects.

Conclusions—Therapeutic concentrations of UFH are associated with platelet activation. (Circulation. 1998;97:251-256.)

Key Words: platelets ▪ cell adhesion molecules ▪ glycoproteins ▪ heparin

The indications for anticoagulant therapy have expanded to include the acute phase of coronary syndromes, resulting in a soaring use of heparin in clinical practice. Simultaneously, new anticoagulants have been developed, including LMWHs and direct thrombin inhibitors,1,2 defining a need for a thorough understanding of the effects of these drugs on the mechanisms of blood clot formation. Thus, although many studies documented that in vitro heparin could enhance platelet aggregation,3–9 the drug prolongs bleeding time in vivo,10 inhibits many platelet functions11–14 to create a platelet defect,15,16 and promotes bleeding risk.15

The present study characterized the platelet effects of therapeutic concentrations of UFH and of enoxaparin, a LMWH, and argatroban, a direct thrombin inhibitor. Platelet aggregation was quantified in PRP by light transmission, and the expression of activation-dependent platelet membrane markers was quantified in whole blood by use of flow cytometry.

Methods

Study Drugs

The drugs studied were unfractionated porcine heparin of molecular weight 5000 to 30,000 D (mean, 15,000; purchased from Leo Laboratories); enoxaparin, a LMWH of 3500 to 5500 D (mean, 4500; provided by Rhône-Poulenc Rorer Canada Inc); and argatroban, a direct inhibitor of the catalytic site of thrombin (provided by Texas Biotechnology Corp).

Study Design

In vivo studies were performed in 43 patients admitted for unstable angina. Twenty-seven patients, six women and 21 men 61±11.0 years of age (range, 41 to 84 years), received UFH, and 16 patients, 6 women and 10 men 64±12.7 years of age (range, 32 to 79 years) received enoxaparin. The UFH was administered as an intravenous bolus of 5000 U followed by an infusion at a rate of 1000 U/h titrated after 6, 12, and 18 hours to an activated partial thromboplastin time 2.5 times control values. Enoxaparin was administered subcutaneously at a dose of 1 mg/kg at 12-hour intervals. These doses of UFH and LMWH resulted in plasma antifactor Xa activity ranging between 0.3 and 0.6 U/mL. All patients received aspirin, but none had had a previous exposition to heparin or to enoxaparin.

Flow cytometric studies were performed in 16 patients administered heparin and in 16 administered enoxaparin, and platelet aggregation studies were done in all patients before the start of the drug and 24 hours later.

For the ex vivo studies, fresh blood was obtained in a fasting state from seven normal healthy volunteers, three women and four men 35±7.5 years of age (range, 24 to 46 years). These volunteers had not received any drugs in the last 14 days prior to the study. The drugs were added to blood obtained 60 minutes after the start of the drug.
been taking any medication in the preceding 2 weeks and had not previously been exposed to heparin therapy. Blood (30 mL) was withdrawn in citrate and immediately divided into four different tubes. Standard heparin was added in one of the aliquots to a concentration of 0.25 U/mL, enoxaparin in another to a concentration of 0.25 U/mL, argatroban in a third to 1 μg/mL, and an equal volume of normal saline as control to the last aliquot.

All blood samples were obtained with a 21-gauge butterfly needle from an antecubital vein. The first 2 mL was discarded. Free-flowing blood was collected in plastic tubes containing 3.8% sodium citrate for volume of normal saline as control to the last aliquot.

Flow Cytometric Assays

Flow cytometric measurements were performed in whole blood by use of a method adapted from Shattil et al.16 and Warkentin et al17 The citrated blood was diluted within 15 minutes of being drawn in a 1:4 ratio with a modified Tyrode’s buffer solution containing NaCl 137 mmol/L, KCl 2.8 mmol/L, MgCl2 1 mmol/L, NaHCO3 12 mmol/L, Na2HPO4 0.4 mmol/L, bovine serum albumin 0.35%, HEPES 10 mmol/L, and glucose 5.5 mmol/L. Then 13 samples were incubated for 30 minutes at 26°C without stirring, and a final ratio of 1:9.

Table 1. Activated GP IIb/IIIa (PAC-1) and P-Selectin (CD62) Expression on Platelet Surface With Infusion of UFH and of LMWH in Patients With Unstable Angina

<table>
<thead>
<tr>
<th></th>
<th>PAC-1</th>
<th>CD62</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+PL %</td>
<td>BI</td>
</tr>
<tr>
<td>Pre-UFH</td>
<td>Basal</td>
<td>2.7 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>ADP</td>
<td>63.8 ± 12.4</td>
</tr>
<tr>
<td></td>
<td>TRAP</td>
<td>10.1 ± 6.1</td>
</tr>
<tr>
<td>UFH</td>
<td>Basal</td>
<td>4.4 ± 3.4*</td>
</tr>
<tr>
<td></td>
<td>ADP</td>
<td>71.5 ± 12.6†</td>
</tr>
<tr>
<td></td>
<td>TRAP</td>
<td>16.2 ± 13.3*</td>
</tr>
<tr>
<td>Pre-LMWH</td>
<td>Basal</td>
<td>2.1 ± 1.9</td>
</tr>
<tr>
<td></td>
<td>ADP</td>
<td>61.0 ± 17.1</td>
</tr>
<tr>
<td></td>
<td>TRAP</td>
<td>15.1 ± 11.2</td>
</tr>
<tr>
<td>LMWH</td>
<td>Basal</td>
<td>2.0 ± 2.1</td>
</tr>
<tr>
<td></td>
<td>ADP</td>
<td>57.1 ± 14.5</td>
</tr>
<tr>
<td></td>
<td>TRAP</td>
<td>11.8 ± 11.0</td>
</tr>
</tbody>
</table>

PL % indicates percent positive platelets; BI, binding index. Data are mean ± SD.

*pP<.05, †P<.01, ‡P<.001 vs pre-UFH; §P<.01 vs pre-LMWH.
Platelet counts were also unchanged after the addition of UFH in vitro (197±24×10^6 per 1 mL before and 195±23×10^6 after), LMWH (197±24×10^6 and 207±23×10^6 per 1 mL), and argatroban (197±24×10^6 and 198±23×10^6 per 1 mL, respectively).

**In Vivo Studies**

Table 1 provides the results of the flow cytometric studies in patients with unstable angina administered UFH or LMWH, and Fig 1 the individual responses. UFH consistently resulted in expression of activated GP IIb/IIIa receptor and of P-selectin. This state of activation was manifested in the basal state, with a nearly twofold increase in the number of activated platelets and in the binding index. In the presence of heparin, platelets were also more sensitive to agonist stimulation with ADP, with a 12% increase in the number of platelets expressing activated GP IIb/IIIa (P<.001) and a 67% increase in the number of platelets expressing P-selectin (P<.001). The respective increases with TRAP were 60% (P<.05) and 65% (P<.001). No activation could be detected with LMWH in the basal state; TRAP stimulation, however, was associated with a 65% increase in the number of platelets expressing P-selectin.

Platelet aggregation increased also twofold during the infusion of heparin. Enoxaparin also increased platelet aggregation; the increase was modest, however, and not statistically significant (Fig 2). Examples of platelet aggregation to ADP and to TRAP before and during an infusion of UFH in a patient with unstable angina are shown in Fig 3.

**In Vitro Studies**

The results of the in vitro studies are provided in Table 2, and the individual data points are illustrated in Fig 4. Again, UFH resulted in detectable platelet activation in all individuals studied. The number of platelets binding PAC-1 increased twofold in the basal state, twofold after TRAP stimulation (P<.01), and by 9.3% after ADP stimulation (P<.05). P-selectin expression increased by 57% in the basal state, by 25% after the addition of TRAP, and by 30% after the addition of ADP. By contrast, enoxaparin and argatroban resulted in no increases in these markers of activation. Enoxaparin slightly but significantly decreased P-selectin expression.

The results of the platelet aggregation studies were consistent (Fig 5), with more than a twofold increase in platelet aggregation after administration of UFH, but no significant changes were noted with enoxaparin and with argatroban. Argatroban nonsignificantly reduced platelet aggregation induced by ADP.

**Discussion**

This study documents that therapeutic concentrations of UFH activate platelets in vivo and enhance platelet aggregability.
The results observed with flow cytometry and with platelet aggregation studies were very consistent. They were also highly reproducible between individuals, both in vivo, in patients with unstable angina and ex vivo, in normal control subjects. No such pro-proaggregant effects were detected with enoxaparin, an LMWH; argatroban, a direct thrombin inhibitor, had no platelet effects.

Flow cytometry provides a sensitive and direct means to detect surface changes on single platelets. Platelet activation, notwithstanding the stimulus, leads to a configurational change in the integrin membrane receptor GP IIb/IIIa, making it competent to bind fibrinogen and other ligands. PAC-1 is a specific antibody binding the activated expression of the receptor. Platelet activation also results in translocation of P-selectin from the alpha granules to cell surface, where it was detected by a specific monoclonal antibody, anti-CD62. In this study, UFH resulted in detectable activation of the GP IIb/IIIa receptor and expression of P-selectin as manifested by an increase in the total number of activated circulating platelets and a greater binding index. The increase in the total amount of PAC-1–positive circulating platelets was 1.7% in the absence of agonists. This increase was small but statistically significant, suggesting that it could have clinical importance. This pool of activated circulating platelets increased fourfold in the presence of low concentrations of agonists, from 1.7% to 7.7% with ADP and from 1.7% to 6.1% with TRAP. ADP is released after platelet stimulation, and TRAP mimics the effects of thrombin on the platelet receptor, suggesting that the stimulation with heparin could be important at sites of endothelial injury at which most of the endogenous platelet stimulation occurs.

**Heparin-Platelet Interactions**

The interactions between heparin and platelets are complex and only partially known. A stimulating effect was observed in this study in normal individuals as well as in patients, suggesting a true physiological effect. Heparin binds to platelet surface to modify responsiveness. Experimental studies with different concentrations and sources of heparin and different agonists have variously documented platelet activation or platelet inhibition. When rapidly administered intravenously, UFH reduced platelet counts and prolonged bleeding times, creating a platelet defect. In vitro, low doses of heparin are more apt to reduce platelet aggregation, and high doses are more likely to increase it. The proaggregant response is detected with most agonists, including ADP, adrenaline, collagen, and the platelet activating factor. It is more consistently observed with low concentrations of ADP (in the range of 0.15 μmol/L) and less consistently with higher concentrations (3 and 5 μmol/L). Similar differential results were observed in

![Figure 3. Typical examples of platelet aggregation to low concentrations of ADP (A) and of TRAP (B) before and during an infusion of UFH in a patient with unstable angina.](image)

| Table 2. Activated GP IIb/IIIa (PAC-1) and P-Selectin (CD62) Expression on Platelet Surface After Addition of UFH, LMWH, and Argatroban in Whole Blood |
|-----------------|------------|-----------------|------------|-----------------|
|                  | PAC-1      | CD62            |
|                  | PL %       | BI              | PL %       | BI              |
| Control          | Basal      | 2.1±0.7         | 0.07±0.03  | 0.7±0.2         | 0.01±0.00       |
|                  | ADP        | 70.8±14.7       | 3.80±1.61  | 11.0±4.4        | 0.19±0.08       |
|                  | TRAP       | 16.4±14.7       | 0.67±0.56  | 2.7±1.7         | 0.04±0.03       |
| UFH              | Basal      | 4.6±2.2*        | 0.17±0.11  | 1.2±0.4*        | 0.02±0.1*       |
|                  | ADP        | 77.4±11.6*      | 4.6±1.99*  | 14.2±4.9†       | 0.25±0.09*      |
|                  | TRAP       | 28.3±23.4†      | 1.2±1.10*  | 3.4±1.9†        | 0.06±0.03*      |
| LMWH             | Basal      | 2.2±2.1         | 0.09±0.07  | 0.5±0.03†       | 0.01±0.00       |
|                  | ADP        | 70.3±7.1        | 3.65±1.85  | 10.7±5.0        | 0.19±0.13       |
|                  | TRAP       | 21.9±14.7       | 1.0±0.74   | 2.7±1.4         | 0.05±0.03       |
| ARG              | Basal      | 2.3±0.7         | 0.09±0.02  | 0.8±0.2         | 0.01±0.00       |
|                  | ADP        | 69.1±19.6       | 3.55±1.75  | 11.4±4.4        | 0.21±0.08       |
|                  | TRAP       | 21.0±14.2       | 0.88±0.56  | 2.5±1.1         | 0.05±0.02       |

Abbreviations as in Table 1, and ARG indicates argatroban a direct thrombin inhibitor. Data are mean±SD. *P<.05, †P<.01, ‡P<.001 vs control.
our laboratory with low and high concentrations of TRAP; the stimulation observed at low concentrations was not reproduced at the higher concentrations of 2.5 to 10 μmol/L. These findings suggest that the proaggregant effect of UFH is modest and can be overcome with strong platelet stimulation. These findings also could possibly explain why the proaggregant action was detected in most studies only at supratherapeutic concentrations of heparin. The platelet stimulation induced by heparin can be blocked by EDTA and by increasing platelet cyclic adenosine monophosphate.24,25 It does not require fibrinogen because it occurs in washed platelet preparations.9 It is not inhibited by blocking cyclooxygenase and does not require ADP.8 In the presence of antithrombin III, heparin inhibits all platelet activities induced by thrombin, including secretion, increases in cytosolic calcium, and aggregation.7 The thrombin receptor peptide used in this study mimics the effects of thrombin on the receptor,26 yet its platelet effects were potentiated and not inhibited by heparin. This observation is consistent with the indirect effect of heparin to inhibit thrombin-induced platelet aggregation, requiring a cofactor. Because the heparin–antithrombin III complex has limited effects on thrombin bound to fibrin,27 heparin may stimulate platelets within or in the vicinity of the blood clot to paradoxically entertain local thrombogenic stimulation. Although circulating thrombin is inactivated by antithrombin III, fibrin-bound thrombin is relatively inaccessible to inhibition by the heparin–antithrombin III complex. Therefore, platelet activation, although weak systematically and counteracted by the anticoagulant effect, may become very significant at the site of thrombus formation.

Enoxaparin and Argatroban

The platelet effects of heparin vary with the molecular weight of heparin and with the affinity for antithrombin III; similarly, the hemorrhagic and antithrombotic properties of the various heparins can be dissociated.1,3,10,28,29 LMWHs with a low affinity for the antithrombin stimulate platelet and high-affinity LMWHs lose their sparing effect when antithrombin III is removed from the plasma.8,30,31 These observations suggest a preferential binding site of heparin for antithrombin III and a less avid site for binding platelets. The binding of LMWH with antithrombin III can therefore minimize the platelet effects. Such is not the case for the high-molecular-weight fractions with an excess of binding sites. The absence of a detectable effect of enoxaparin in our study, except after stimulation with TRAP, which is a strong agonist, is compatible with this concept. Different responses could be observed with other LMWHs with a different affinity for antithrombin III or with different doses. Argatroban is a direct thrombin inhibitor requiring no cofactor for its effects. It was not associated with any detectable platelet activity in this study.

Study Limitations

An alternative explanation for the platelet activation detected in this study could be the unstable state of the patients. Unstable angina can be associated with an elevation of platelet factor 4 and thromboglobulin,32,33 increased production of thromboxane A2,34 and thrombin generation.35 This explanation is unlikely in our study because no activation was detected in the acute phase before the initiation of heparin and because all patients were stable on treatment. Furthermore, the activation was reproducible in the heparin patients and absent in the two other groups without standard heparin. Samples in the study were also performed at the same hour of the day to minimize the effects of circadian variation in platelet function and in coagulant activity.30,36 An artifactual platelet activation related to blood manipulation is also unlikely, considering the reproducibility of the results before and during the experiments.

Figure 4. Percent FITC-positive platelets (PL %) to PAC-1 (activated GP IIb/IIIa) and to CD62 (P-selectin) before and after addition of UFH in whole blood to a concentration of 0.25 U/mL. Each line represents the data of one normal individual.

Figure 5. Maximum (max) platelet aggregation in PRP in normal individuals after the addition in whole blood of normal saline (control), UFH, enoxaparin (LMWH), and argatroban (ARG). Platelet aggregation to low concentrations of ADP and TRAP is significantly increased in the presence of UFH. *P<.05, **P<.01 vs control.
after heparin and before and after enoxaparin. Each patient served as his or her own control in the in vivo study, and the ex vivo studies were performed by addition of heparin, enoxaparin, or argatroban in blood obtained from one single vein puncture.

Significance of Results
The platelet effects of heparin have received relatively little attention in clinical practice except in the syndromes of heparin-induced thrombocytopenia. This situation occurs ≈5 days after the onset of therapy or earlier in patients previously exposed to heparin. None of the normal volunteers in this study had previously received heparin, and patients with unstable angina were studied early after the initiation of heparin and had normal platelet counts. The platelet stimulating effects observed with UHFI were only modest, and the clinical relevance of the observation is unknown. This study was not designed to study the consequences of the platelet stimulation. The proaggregant effects, however, were reproducible and much amplified in the presence of ADP and of TRAP, suggesting that they could be important at the site of thrombus formation at which platelet stimulation occurs. It can be hypothesized that the platelet effect of standard heparin could lead to paradoxical stimulation of thrombosis in some clinical circumstances and contribute to the therapeutic failure of heparin in conditions such as failure of reperfusion and infarct extension in acute myocardial infarction and refractory ischemia and rebound reactivation after heparin discontinuation in unstable angina. Prevention of this platelet activation could therefore enhance the antithrombotic potential of heparin and its clinical effectiveness. Aspirin used in patients with unstable angina in this study did not prevent the activation. The more potent inhibitors of platelet aggregation, such as the inhibitors of the platelet membrane receptor GP IIb/IIIa, may have some effects; characterization of their effects in the presence and absence of heparin may help researchers understand some of the mechanisms for their clinical benefits. Alternatively, the use of LMWH or of a direct thrombin inhibitor could be advantageous. Knowledge of the exact site of the heparin effects on platelets could also be of help in the design of target specific therapy.

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References
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