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

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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, 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, the drug prolongs bleeding time in vivo, inhibits many platelet functions, and promotes bleeding risk.

The present study characterized the platelet effects of therapeutic concentrations of UFH and of enoxaparin, a LMWH, and of 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).
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 from an antecubital vein. The first 2 mL was discarded. Free-flowing 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 and Warkentin et al. 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, pH 7.4. Then 13 mL of diluted blood was divided into Eppendorf tubes containing 5 μL of a saturating concentration of antibodies and 12 mL of a synthetic pure TRAP (amino acid sequence, SFLRNPDPHDKYEPF; provided by Dr Beat Steiner, Hoffmann-La Roche) and of ADP (Sigma Chemical Co) dissolved in Tyrode’s buffer solution. The final concentrations of TRAP and of ADP were 0.625 and 0.312 μmol/L, respectively. The samples were incubated for 30 minutes at 26°C without stirring, and further reaction stopped by addition of 500 μL of 1% formaldehyde in L of 1% formaldehyde in 106 per 1 mL before and 196 67 PAC-1 CD62 were conjugated with FITC and CD42b with PE. Negative murine IgG monoclonal antibodies were also used to assess nonspecific blocking.

A total of 5000 platelets from each fixed sample were analyzed within 1 hour of sampling with a Coulter EPICS XL flow cytometer (Coulter Corp). This flow cytometer is equipped with a 100-mW argon laser to produce a laser beam at 488 nm, detecting FITC and PE fluorescence at band-pass filters of 525 and 575 nm, respectively. The platelet population was identified by forward scatter for cell size and by side scatter for cell granularity. Double-labeling experiments identified >97% of the particles within the area of interest as positive for CD42b binding. The percentage of fluorescence positive platelets (PL%) and a binding index calculated as mean fluorescence intensity per particle times PL%/100 were obtained in duplicate from the instrument computer system.

**Platelet Aggregation Studies**

Platelet aggregation was evaluated in PRP by use of a four-channel light transmission aggregometer (Chronolog Corp). The PRP was prepared by centrifugation of whole blood at 1000 rpm for 10 minutes, and the platelet-poor plasma was prepared by centrifugation of the remaining blood at 3000 rpm for an additional 10 minutes. PRP served to set 0% light transmission; platelet-poor plasma, 100% transmission. Platelet aggregation was measured 10 minutes after the addition of TRAP 0.625 μmol/L and of ADP 0.312 μmol/L (final concentration).

**Statistical Analyses**

The means of duplicate measurements obtained before and after drugs were compared by Student’s paired t tests. Results are expressed as mean±SD. A value of P<.05 was considered significant.

**Results**

**Platelet Count and Activated Thromboplastin Time**

Baseline platelet counts were within the normal range in all patients and unchanged after the intravenous administration of UFH (201±67×10⁶ per 1 mL before and 196±19×10⁶ per 1 mL after) and of LMWH (221±91×10⁶ and 233±98×10⁶ per 1 mL, respectively). Heparin prolonged the activated thromboplastin time values from 25.8±2.55 to 51.6±13.26 seconds.
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 receptor and of 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-thrombotic 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

| 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         | 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 repro-
duced 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 content.24,25 It does not 
require fibrinogen because it occurs in washed platelet prepa-
rations.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 aggre-
gation.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. Al-
though 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 hepa-
rins 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 
oberved with other LMWHs with a different affinity for anti-
thrombin 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. Samplings 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
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 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 UFH 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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