

BASIC RESEARCH

Distinct yet complementary mechanisms of heparin and glycoprotein IIb/IIIa inhibitors on platelet activation and aggregation: implications for restenosis during percutaneous coronary intervention

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Objective: To study the effect of unfractionated heparin (UFH) versus low molecular weight heparin (LMWH) in combination with glycoprotein (Gp) IIb/IIIa blockers on platelet activation and aggregation.

Methods: Washed platelets were stimulated with thrombin in the presence or absence of UFH (monoparin), LMWH (enoxaparin), and a Gp IIb/IIIa blocker (abciximab, eptifibatide, or tirofiban).

Results: Although Gp IIb/IIIa antagonists blocked the final common pathway of thrombin induced platelet aggregation, UFH and LMWH were better at blocking upstream platelet activation. UFH was significantly more effective than LMWH at inhibiting P selectin expression ($p = 0.001$) and platelet derived growth factor release from thrombin activated platelets ($p = 0.012$).

Conclusions: UFH and LMWH exert complementary effects to Gp IIb/IIIa blockers by inhibiting afferent pathways of platelet activation. Coadministration of heparin with Gp IIb/IIIa blockers provides improved protection against persistent platelet activation, thereby improving outcome after percutaneous coronary intervention. Judging from these data, UFH may be more effective in this regard than LMWH, at least in vitro. The use of LMWH in preference to UFH during percutaneous coronary intervention, although initially attractive, may inadequately protect against platelet activation despite the presence of Gp IIb/IIIa blockers.

Platelet activation is a key feature of acute coronary syndromes (ACS)¹ and also occurs after percutaneous coronary intervention (PCI), in particular when a stent is placed.² Periprocedural use of platelet glycoprotein (Gp) IIb/IIIa ($\alpha_{IIb}\beta_{IIIa}$) receptor (Gp IIb/IIIa) blockers has been shown to reduce the risk of major adverse cardiac events (death, myocardial infarction, and repeat revascularisation) after PCI with or without coronary stenting.³⁻⁴ Use of Gp IIb/IIIa blockers has also been shown to reduce event rates in patients with ACS.⁵⁻⁸ Furthermore, combined use of Gp IIb/IIIa antagonists and low dose heparin reduces the risk of ischaemic complications, without increasing the risk of haemorrhage.

Long term restenosis of the dilated segment of a coronary artery remains a problem and occurs in up to 30% of patients after PCI even with the use of stents.⁹⁻¹⁰ Greater activation of inflammatory processes after PCI predicts restenosis, perhaps by stimulating smooth muscle cell proliferation.¹¹⁻¹² Restenosis results from a combination of smooth muscle proliferation, recoil, and incorporation of thrombus⁹⁻¹³⁻¹⁴ and has remained a problem despite the use of Gp IIb/IIIa receptor blockers.³ Persistent platelet activation, despite the abrogation of aggregation by the Gp IIb/IIIa blockers, may play a key part through the generation of platelet-leucocyte conjugates, increased leucocyte activation, and release of inflammatory mediators and growth factors.¹¹⁻¹⁵⁻¹⁶ P selectin, an adhesion molecule, acts as a marker for activated platelets, which contribute to leucocyte conjugate formation by binding P selectin glycoprotein ligand (PSGL)-1.¹⁷

At present, before PCI, a bolus of unfractionated heparin (UFH) is given, with or without additional Gp IIb/IIIa blockade. The main limitation of UFH results from its propensity to bind to positively charged proteins and surfaces. Pharmacokinetic limitations are caused by binding of UFH to plasma proteins, platelet proteins, and endothelial

cells, resulting in a variable anticoagulant response and the phenomenon of heparin resistance. Although an exact therapeutic dose of low molecular weight heparin (LMWH) required for PCI is still unknown, it has been suggested as an alternative, since it has a predictable dose response, eliminating the need for assessments of coagulation. In addition, the risk of heparin induced thrombocytopenia is lower with LMWH.¹⁸ Data from treatment of ACS suggest benefit in using LMWH rather than UFH.¹⁹⁻²⁰ Therefore, it has been suggested that use of LMWH in preference to UFH in PCI may be beneficial even though randomised controlled comparisons are not yet available.

The two main thrombin receptors on human platelets are protease activated receptor (PAR)-1²¹ and platelet Gp Ib.²² These receptors act synergistically in the platelet response to thrombin through a necessary cofactor role for Gp Ib during PAR-1 activation.²³ Activation of Gp Ib by thrombin, in turn, is inhibited by heparin and this is directly proportional to the chain length of the oligosaccharide.²⁴ The possibility, therefore, exists that heparin may provide additional protection beyond anticoagulation in PCI by inhibiting platelet activation and that protection is related to the molecular weight of the molecule.

To understand better the relative merits of using combinations of Gp IIb/IIIa antagonists with UFH and LMWH to

Abbreviations: ACS, acute coronary syndromes; ELISA, enzyme linked immunosorbent assay; ESSENCE, efficacy and safety of subcutaneous enoxaparin in non-Q wave coronary events; Gp, glycoprotein; LMWH, low molecular weight heparin; PAR, protease activated receptor; PCI, percutaneous coronary intervention; PDGF, platelet derived growth factor; POLONIA, Polish-American local Lovenox NIR assessment; PSGL, P selectin glycoprotein ligand; SYNERGY, superior yield of the new strategy of enoxaparin, revascularisation and glycoprotein IIb/IIIa inhibitors; TRAP, thrombin receptor activating peptide; UFH, unfractionated heparin

control platelet function, we have analysed platelet activation and aggregation *in vitro*. Our results confirm that heparin provides protection from thrombin induced platelet activation not afforded by Gp IIb/IIIa antagonists and in addition that UFH may be significantly better in this respect than LMWH.

METHODS

Reagents and antibodies

Collagen, thrombin, and ADP were obtained from Sigma Diagnostics (Poole, UK). Adrenaline was obtained from Helena Biosciences (Sunderland, UK). The PAR-1 agonist hexapeptide SFLLRN (thrombin receptor activating peptide (TRAP)-6) was synthesised by the Advanced Biotechnology Centre (Imperial College School of Medicine, London, UK). Abciximab (ReoPro) was obtained from Centocor BV (Leiden, the Netherlands), tirofiban (Aggrastat) from Merck Pharmaceuticals (Middlesex, UK), eptifibatid (Integrilin) from Millennium Pharmaceuticals (Cambridge, UK), enoxaparin (Clexane) from Rhône-Poulenc Rorer (Kent, UK), and UFH sodium (Monoparin) from CP Pharmaceuticals Ltd (Wrexham, UK). Phycoerythrin conjugated monoclonal mouse anti-human CD62P P selectin and mouse IgG₁ negative control antibodies were purchased from Dako A/S (Ely, UK). The enzyme linked immunosorbent assay (ELISA) kit for platelet derived growth factor (PDGF)-AB was purchased from R&D Systems (Abingdon, UK).

Platelet preparation

Washed platelets were prepared from peripheral blood samples of normal healthy human volunteers, as previously described.²⁵ Absence of clotting factors was shown by lack of detectable antithrombin III (< 0.01 U/ml), protein C (< 0.01 U/ml), and protein S (< 0.01 U/ml) in the platelet suspension.

Platelet aggregation

Platelet aggregation was studied with the use of an APACT 4 platelet aggregometer (Helena Biosciences). Platelet agonists such as thrombin (3 nmol/l), TRAP-6 (50 µmol/l), adrenaline (10 µg/ml), collagen (50–100 µg/ml), and ADP (4 µmol/l) were added to 200 µl aliquots of platelet solution (200 × 10⁹–400 × 10⁹/l), as previously described,²⁵ with combinations of abciximab (3.5 µg/ml), tirofiban (0.25 µg/ml), or eptifibatid (2.5 µg/ml) and LMWH (0.25 U/ml) or UFH (0.25 U/ml). The concentrations of UFH (mean molecular weight 15 000 Da) and LMWH (mean molecular weight 4500–5000 Da) were based on reported therapeutic plasma concentrations²⁶ and those of Gp IIb/IIIa inhibitors on the clinical doses used in a 70 kg patient with a circulating volume of 5 l.

Flow cytometric analysis

Platelets were washed and aggregation was initiated by agonists as described above. At fixed time points, based on the aggregation parameters (thrombin 180 seconds, collagen 300 seconds, ADP 180 seconds, adrenaline (epinephrine) 300 seconds) the platelets were fixed with 1% paraformaldehyde, washed with phosphate buffer solution, and centrifuged at 3000 rpm. The pellets were resuspended and incubated for 30 minutes with 20 µg/ml of either phycoerythrin conjugated monoclonal mouse anti-human P selectin (CD62P) antibody or phycoerythrin conjugated mouse IgG₁ negative control antibody. The platelets were washed again, centrifuged at 3000 rpm, and resuspended in 1 ml of phosphate buffer solution. P selectin expression was analysed with a Beckman Coulter XL flow cytometer (High Wycombe, UK) on the gated platelet population. P selectin was expressed as relative fluorescence intensity, which was obtained by dividing the mean fluorescence intensity obtained with phycoerythrin

conjugated CD62P antibody by that obtained with phycoerythrin conjugated control antibody.

Intraplatelet calcium concentrations

Platelets were loaded with Fura-2 AM (Molecular probes Inc, Eugene, Oregon, USA) for 45 minutes at 37°C, as previously described.²⁵ Intracellular calcium was measured by measuring the Fura-2 fluorescence at 340 nm excitation and 510 nm emission with an LS 50 B spectrofluorometer (Perkin Elmer, Beaconsfield, UK). Platelets prepared with UFH (0.2 U/ml) or abciximab (2 µg/ml) were added until a steady baseline reading was obtained. Platelet agonists were then added at the same concentrations as those used in aggregometry, after which fluorescence tracings were obtained. Intraplatelet calcium fluxes were determined by subtracting the baseline concentration from peak concentrations and calibrating to a known standard.

Statistical analysis

Treatment groups in CD62P, PDGF, and monocyte chemo-attractant protein 1 experiments were compared by a two tailed Student's *t* test. Significance was assumed at *p* < 0.05.

RESULTS

Selective versus broad based inhibition of platelet aggregation by heparin and Gp IIb/IIIa inhibitors, respectively

As expected, we showed that platelet aggregation caused by thrombin, adrenaline, collagen, or ADP was inhibited by abciximab through blockade of the Gp IIb/IIIa receptor (fig 1). Similar results were obtained with eptifibatid and tirofiban (data not shown). In contrast, UFH and LMWH inhibited aggregation caused by thrombin (fig 1A) but not caused by adrenaline (fig 1B), collagen (fig 1C), or ADP (fig 1D). To confirm that heparin was acting on the afferent arm of the thrombin activation pathway, we showed that transduction of intraplatelet activating signals, as measured by calcium flux, was inhibited by UFH (fig 2A). In contrast, when the thrombin receptor PAR-1 was activated directly by TRAP-6, which bypasses the need for cofactoring with Gp Ib,²³ UFH and LMWH had no effect on calcium flux (fig 2B) or platelet aggregation (fig 2C). This was therefore consistent with heparin inhibiting the thrombin induced platelet activation by blocking the platelet Gp Ib receptor.²⁴

Selective inhibition of platelet activation by heparin but not Gp IIb/IIIa blockers

To examine downstream consequences of platelet activation, we investigated P selectin (CD62P) expression and release of PDGF from activated platelets. Using P selectin expression as a marker of platelet activation, we showed that thrombin caused platelet activation and that this was significantly inhibited by LMWH (*p* = 0.0183) and UFH (*p* < 0.001) (fig 3A). Furthermore, UFH was significantly better than LMWH (*p* = 0.0014) at blocking P selectin expression. None of the Gp IIb/IIIa antagonists significantly inhibited P selectin expression caused by thrombin, nor did they act synergistically when combined with LMWH or UFH (fig 3B, C, D). An analogous picture was observed in the case of PDGF release by platelets. Here also, thrombin caused PDGF release and this was significantly inhibited by LMWH (*p* = 0.0300) and UFH (*p* = 0.0121) (fig 4A). Similarly, none of the Gp IIb/IIIa blockers significantly inhibited PDGF release and there was no additive effect when combined with LMWH or UFH (fig 4B, C, D). Taken together, these results show that UFH and LMWH protect platelets from activation by thrombin but that the Gp IIb/IIIa antagonists abciximab, eptifibatid, and tirofiban do not.

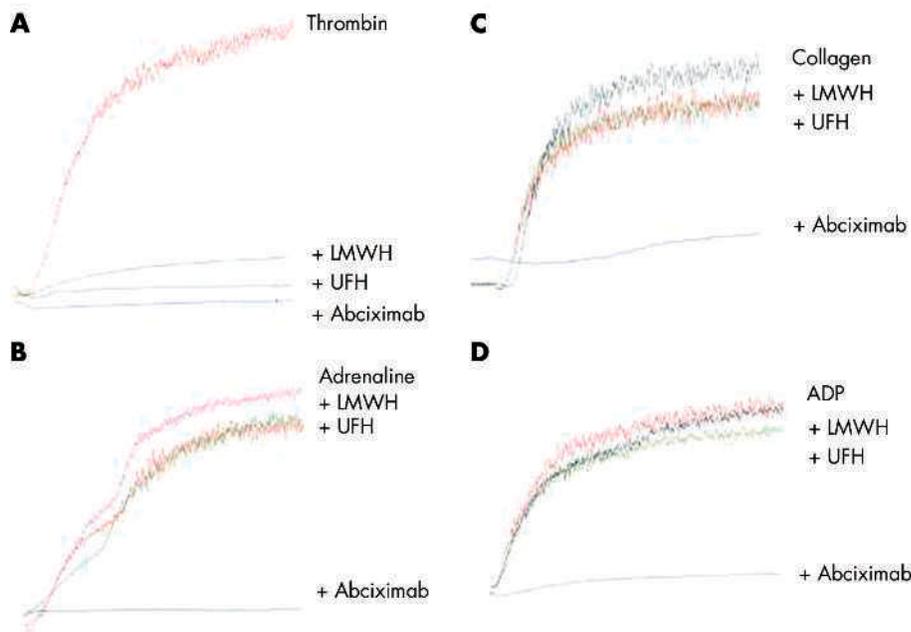


Figure 1 Platelet aggregometry traces showing effects of clinically relevant concentrations unfractionated heparin (UFH), low molecular weight heparin (LMWH), and abciximab on platelet aggregation induced by (A) thrombin, (B) adrenaline, (C) collagen, and (D) ADP. Washed platelets (4×10^7 – 8×10^7) were preincubated with UFH, LMWH, or abciximab for five minutes before addition of agonist. Aggregation was carried out for 300 seconds. Traces are representative of 10 similar experiments.

DISCUSSION

Prevention of thrombin induced platelet activation reduces acute complications after PCI. UFH and LMWH inhibit thrombin induced platelet activation, as judged by platelet aggregation, P selectin expression, and PDGF release. However, heparin is effective only when thrombin is the agonist. In contrast, Gp IIb/IIIa inhibitors block aggregation caused by all platelet agonists but have no effect on P selectin expression or PDGF release. The actions of heparin and Gp

IIb/IIIa antagonists are, therefore, complementary. In addition, we have found that complete inhibition of platelet activation was afforded only by UFH.

The use of Gp IIb/IIIa blockers in ACS and during PCI is validated by clinical trial data.^{5–8} The clinical benefits of LMWH over UFH have been established in patients with ACS but not in PCI.^{19–20} This has not stopped suggestions that LMWH pharmacotherapy in PCI may be as safe and effective as UFH.^{27–29} The present study, however, suggests that UFH is more effective than LMWH in inhibiting platelet activation caused by thrombin.

Persistent platelet activation, despite the abrogation of aggregation by the Gp IIb/IIIa blockers, may play a key part in restenosis after PCI through the generation of platelet–leucocyte conjugates, increased leucocyte activation, and the release of inflammatory mediators and growth factors.^{11–16} Platelet–leucocyte conjugates are increased in ACS and this has been shown to be due to the molecular interaction between increased P selectin on platelets and PSGL-1 expressed by leucocytes.^{17–30} Thrombin activated platelets use the P selectin/PSGL-1 adhesion pathway to stimulate conjoined monocytes, thus leading to secretion of the proinflammatory cytokines interleukin 1 β , interleukin 8, and monocyte chemoattractant protein 1.^{31–32} P selectin also induces tissue factor expression and fibrin deposition by monocytes, thus contributing to the evolution of the thrombus.^{33–34} Since UFH exhibited substantially greater inhibition of P selectin expression than LMWH, this may translate into decreased inflammation and thrombus formation and ultimately lower restenosis rates,¹⁵ if UFH is used rather than LMWH during PCI. Gp Ib binding by thrombin is inhibited by heparin and the extent of inhibition was directly related to the oligosaccharide chain length.²⁴ Since under normal conditions PAR-1 and Gp Ib act synergistically in the platelet response to thrombin,²³ this may explain why UFH is more effective than LMWH at blocking platelet activation.

Three small trials have compared LMWH with UFH during PCI without Gp IIb/IIIa blockers.^{35–37} Although the POLONIA (Polish-American local Lovenox NIR assessment) study suggested a possible reduction in restenosis with local delivery of enoxaparin before PCI, these results have yet to be confirmed.³⁷ Data from the ESSENCE (efficacy and safety

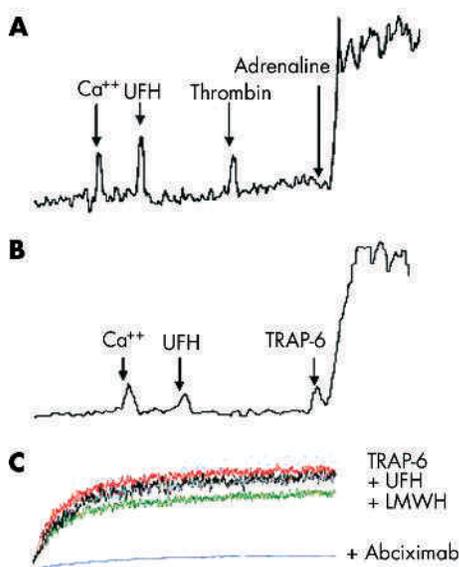


Figure 2 (A) UFH inhibited thrombin but not adrenaline induced platelet calcium fluxes. (B) UFH did not affect thrombin receptor activating peptide (TRAP)-6 induced calcium fluxes. (C) Abciximab, but not UFH or LMWH, inhibited TRAP-6 induced platelet aggregation. Washed platelets (4×10^7 – 8×10^7) were preincubated with UFH, LMWH, or abciximab for five minutes before addition of agonist. Aggregation was carried out for 300 seconds. Traces are representative of 10 similar experiments. Panels (A) and (B) show representative fluorimetry traces indicating calcium influx on an arbitrary scale based on Fura-2 loaded platelets (4×10^7 – 8×10^7). Calcium flux was read over 300 seconds. Traces are representative of five similar experiments.

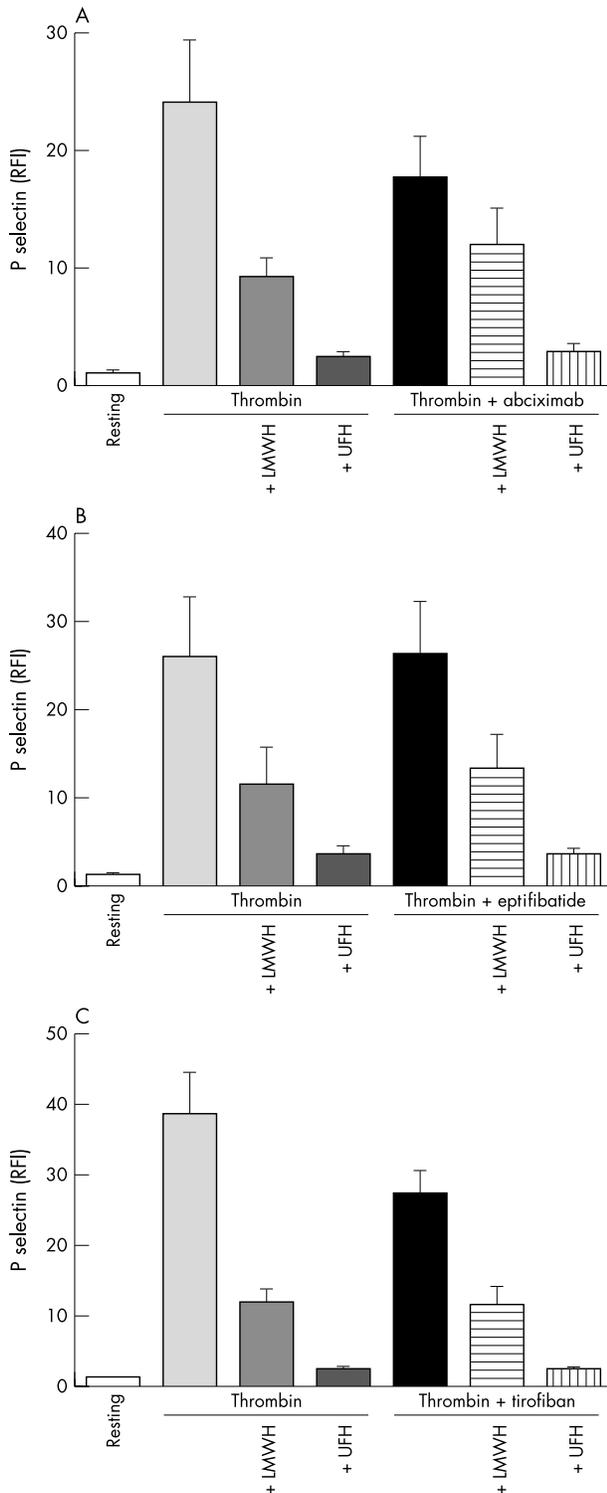


Figure 3 Effect of (A) abciximab, (B) eptifibatide, and (C) tirofiban in combination with UFH or LMWH on thrombin induced P selectin expression. Platelets were prepared as described for aggregation. Platelet activation was halted at 180 or 300 seconds by the addition of paraformaldehyde before staining with anti-P selectin (CD62P) and flow cytometric analysis. Results are depicted as the mean (SEM) relative fluorescent intensity (RFI) from eight experiments.

of subcutaneous enoxaparin in non-Q wave coronary events) trial (comparing LMWH with UFH for ACS) suggests that PCI can be safely performed while the patient is given LMWH. However, only 13% of patients underwent PCI and these

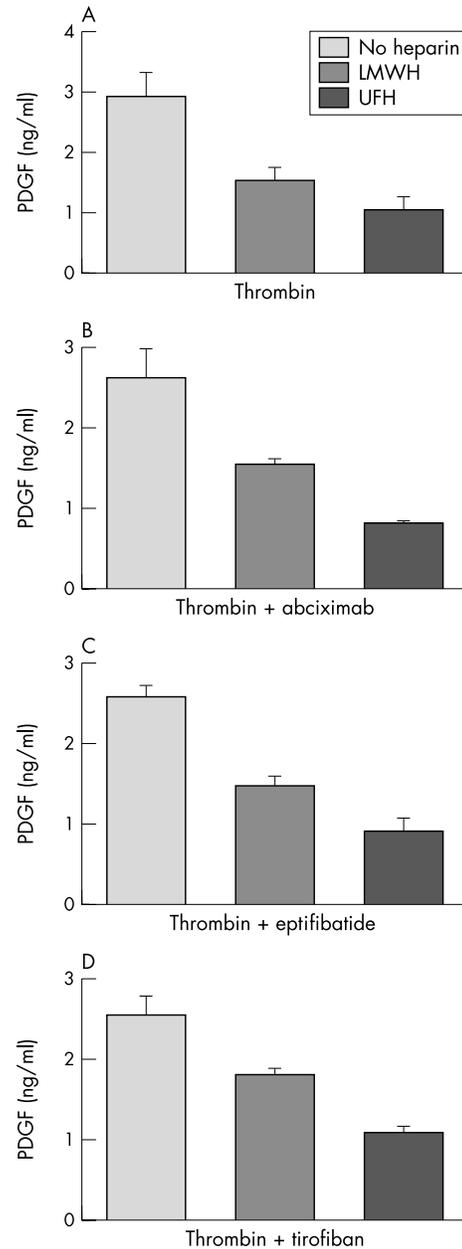


Figure 4 Effect of (A) thrombin, (B) thrombin + abciximab, (C) thrombin + eptifibatide, and (D) thrombin + tirofiban in combination with UFH or LMWH on platelet derived growth factor (PDGF) release. Supernatants were collected from aggregated platelets and PDGF concentrations were determined by enzyme linked immunosorbent assay (ELISA). Supernatants were assayed in quadruplicate. Results are mean (SEM) from two experiments.

patients were not randomly assigned to UFH or LMWH.²⁷ In addition, 56 of the 445 patients given LMWH got extra UFH during the PCI.

Initial safety studies attest to the ability of Gp IIb/IIIa blockers plus LMWH to stop acute thrombosis after PCI.³⁸⁻³⁹ However, no longer term data are available to assess the effect on restenosis. The A-Z trial is assessing the impact of enoxaparin versus UFH in patients with ACS who are already taking tirofiban.⁴⁰ Although the comparison will not be randomised, the majority of patients in the trial are likely to undergo intervention and it may be possible to compare UFH groups with LMWH groups. More direct data will be available

from the SYNERGY (superior yield of the new strategy of enoxaparin, revascularisation and glycoprotein IIb/IIIa inhibitors) trial, in which patients taking Gp IIb/IIIa blocker will be randomly assigned to the use of either enoxaparin or UFH during PCI.⁴¹ Our data provide a framework through which the results of clinical trials may be explained.

We have taken forward early studies^{23, 24} by examining thrombin induced platelet activation based on clinically relevant markers such as P selectin expression and PDGF release and by using clinical concentrations of commercially available heparin and Gp IIb/IIIa inhibitors. We have also confirmed by using TRAP-6 that the likely mechanism of platelet protection is through inhibition of Gp Ib. Our study was undertaken in vitro and the effects shown may not translate into an in vivo effect on restenosis. However, in the absence of any relevant clinical trials, the in vitro approach is useful in assessing mechanisms of action. Although Xiao and colleagues²⁶ have shown that UFH activates resting platelets, the level of activation is insignificant when compared with that caused by thrombin. We have not addressed the combined use of heparin with oral antiplatelet agents such as aspirin and clopidogrel that are used clinically during ACS and PCI. Clopidogrel inhibits platelet aggregation in response to ADP and this may be of additional benefit.⁴²

Conclusion

The present investigation suggests that UFH and LMWH exert complementary effects when coadministered with Gp IIb/IIIa blockers. By inhibiting the afferent arm of thrombin induced platelet activation heparin may provide additional protection beyond anticoagulation in PCI. UFH may be more effective in this regard than LMWH, at least in vitro. Restenosis after PCI may, in part, be caused by smooth muscle cell proliferation related to platelet activation. The use of LMWH in preference to UFH during PCI, although superficially attractive, may inadequately protect against platelet activation despite the presence of Gp IIb/IIIa blockers.

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IMAGES IN CARDIOLOGY

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Successful stenting of the left anterior descending artery in a patient with a single left coronary ostium

A 51 year old patient presented with an acute anterior myocardial infarction and had reperfusion therapy with thrombolysis. Electrocardiograms post-thrombolysis showed complete resolution of ST segment elevation with normal creatine kinase values and troponin T concentration of 0.02 µg/l. Because of post-infarct unstable angina the patient underwent cardiac catheterisation. This showed normal left ventricular function with a single left coronary ostium. The left main stem was normal. The left anterior descending artery (LAD) had two severe subtotal occlusions in the proximal and mid thirds (below left, black arrows). Arising from between these two stenoses was the right coronary artery (RCA) which was non-dominant and diseased with atheroma (white arrow). The left circumflex was normal and dominant. Percutaneous coronary intervention (PCI) was undertaken with stenting to the LAD artery. The final angiographic result was excellent with the RCA

remaining patent and exiting the stented region of the LAD (below right).

An isolated single coronary ostium is a rare coronary anomaly occurring in 0.024–0.04% of the population. When a single coronary ostium arises from the left sinus of Valsalva, the anomalous RCA usually arises from the LAD artery. Other sites of origin are the circumflex artery, pulmonary trunk, aorta or extra cardiac arteries such as the internal mammary or subclavian arteries. PCI through a single coronary ostium is technically possible. These patients are at a higher risk of ischaemic complications as the single coronary ostium is the myocardium's sole supply of blood.

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