

Critical Review

Platelets and Anti-platelet Therapy

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Abstract. Platelets play a central role in the hemostatic process and consequently are similarly involved in the pathological counterpart, thrombosis. They adhere to various subendothelial proteins, exposed either by injury or disease, and subsequently become activated by the thrombogenic surface or locally produced agonists. These activated platelets aggregate to form a platelet plug, release agonists which recruit more platelets to the growing thrombus, and provide a catalytic surface for thrombin generation and fibrin formation. These platelet-rich thrombi are responsible for the acute occlusion of stenotic vessels and ischemic injury to heart and brain. A range of anti-platelet drugs are currently used, both prophylactically and therapeutically, in regimens to manage thrombo-embolic disorders. These include inhibitors of the generation, or effects, of locally produced agonists; several large clinical trials have supported roles for cyclooxygenase inhibitors, which prevent thromboxane generation, and thienopyridine derivatives, which antagonize ADP receptors. Similarly intravenous α IIb β 3 antagonists have been shown to be effective anti-thrombotics, albeit in highly selective situations; in contrast, to date studies with their oral counterparts have been disappointing. Recent advances in understanding of platelet physiology have suggested several novel, if yet untested, targets for anti-platelet therapy. These include the thrombin receptor, the serotonin handling system, and the leptin receptor.

Keywords: platelet, anti-thrombotic, aspirin, α IIb β 3 antagonist

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Introduction

Platelets play a central role in the hemostatic process, including recognizing the site of injury, recruiting additional platelets by intercellular signaling, adhering to each other, and interacting with the coagulation cascade to form a haemostatic plug. Inappropriate platelet activation, and subsequent thrombus formation, are important in the clinical complications of arterial atherosclerosis and thrombosis (1, 2). Therefore the process, and consequences, of platelet activation *in vivo* remains a subject of scrutiny (3), with specific interest in platelets as targets for anti-thrombotic agents (4, 5) (Fig. 1).

Platelet structure

Platelets are derived, and released, from the progenitor megakaryocyte in the bone marrow and have a life-span of 7–10 days. They are the smallest cells in circulating blood and exist as biconvex discs with an equatorial diameter of 2–3 μm . The platelet membrane is an asymmetrical phospholipid bilayer in which phosphatidylcholine is evenly distributed; sphingomyelin found exclusively in the outer leaflet; and high levels of phosphatidylethanolamine, phosphatidylcholine, and phosphatidylinositol in the inner leaflet. A range of proteins are embedded in the platelet membrane notably adhesive, stimulatory, and inhibitory receptors (3).

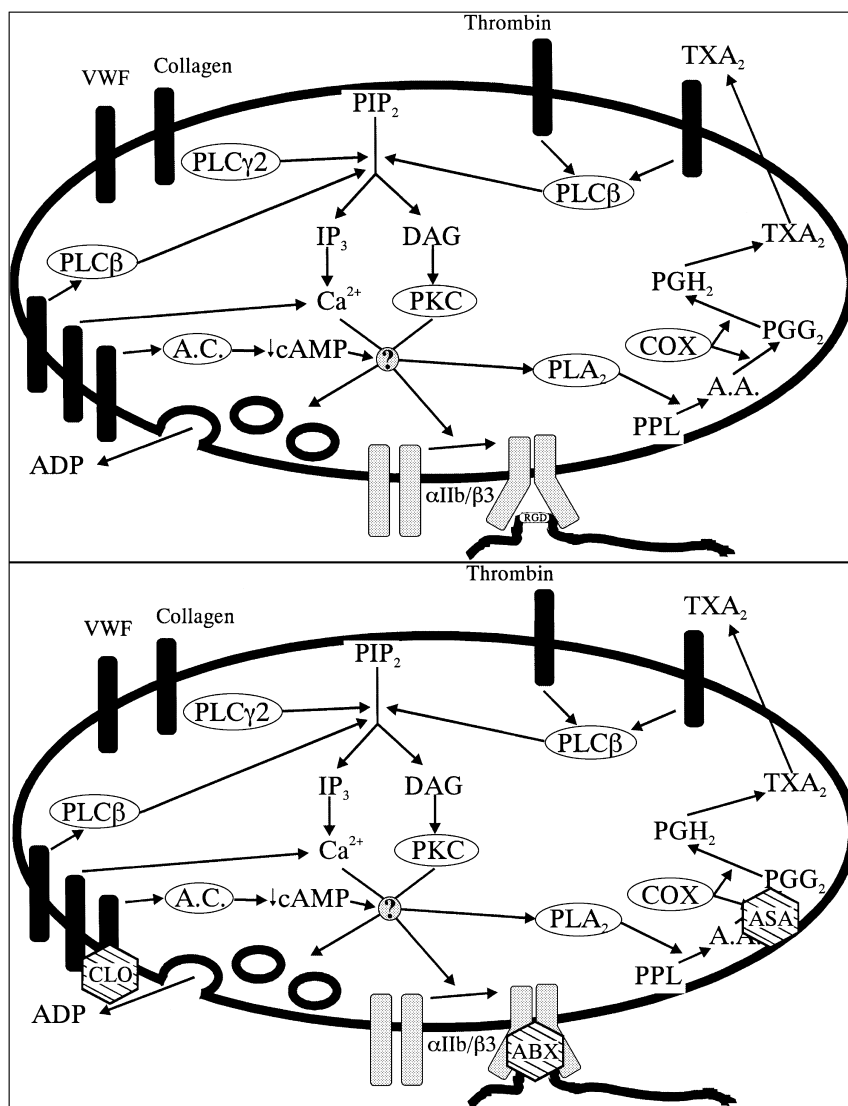


Fig. 1. Mechanisms of agonist-induced platelet activation and site of action of inhibitors. Upper panel: Platelet activation by collagen, thrombin, and ADP including intracellular signaling pathways leading to TXA₂ production, ADP release from dense granules and conformational changes in the $\alpha\text{IIb}/\beta\text{3}$ integrin allowing fibrinogen binding. Lower panel: Sites of action of aspirin (ASA), clopidogrel (CLO), and abciximab (ABX). A.A., arachidonic acid; PPL, phospholipids; A.C., adenylate cyclase; see text for additional abbreviations.

Although anucleate, platelets contain a variety of intracellular organelles, including a circumferential microtubular band, a pair of membrane systems (the dense tubular system and the plasma membrane associated surface connected canalicular system) and three types of secretory granules (α granules, dense granules, lysosomes) (3).

Dense tubular system

The dense tubular system (DTS) is derived from the smooth endoplasmic reticulum found in the parent megakaryocyte (6). The DTS is the major calcium sequestering organelle in platelets and acts to maintain the resting free calcium concentration ($[Ca^{2+}]_i$) of 90 nM (7). Not surprisingly, therefore, the DTS is also the location of a pair of calcium-liberating inositol 1,4,5 trisphosphate (IP_3) receptors (IP_3RI and IP_3RII) (8). As outlined below, IP_3 , generated in response to agonist-induced platelet activation, acts to liberate calcium from the DTS and elevate the $[Ca^{2+}]_i$.

The second major role of the DTS is in arachidonic acid metabolism. Arachidonic acid liberating enzymes phospholipase (PL) A_2 and diglyceride lipase (9) are both found in the DTS, as are the enzymes involved in the stepwise conversion of arachidonic acid to thromboxane (TX) A_2 , prostaglandin (PG) H-synthase-1 (PGHS-1) and TX synthase (10). As outlined below, the generation of TXA_2 is an important mediator of platelet function, and conversely inhibition of this pathway serves as a primary target for anti-platelet therapy.

Granules

Platelets contain three types of granules, the contents of each of which may be released following stimulation. α Granules are most numerous and contain fibrinogen, growth factors (platelet derived growth factor, β -transforming growth factor), and cytokines (platelet factor 4, neutrophil-activating peptide-2, β -thromboglobulin) (11, 12). Dense granules contain ADP, serotonin, and calcium (12, 13), and lysosomal granules contain acid proteases, acid glycosidases, acid phosphatases, and aryl sulphatases (12, 14).

Platelet activation

Platelets initially adhere to both exposed subendothelial collagen and von Willebrand Factor at the site of injury to form a fragile monolayer. This adhesion is mediated by the $\alpha_2\beta_1$ integrin and glycoprotein (GP) VI collagen receptors and GPIb (as a component of the GPIb/V/IX complex), the von Willebrand Factor receptor, on the platelet surface. Platelets are then acti-

vated by a variety of agents which act to recruit additional platelets to the site of injury, leading to the consolidation of the haemostatic plug or aggregate (15). This activation process is initiated by the engagement of a range of specific cell surface receptors (Table 1) and associated intracellular signaling pathways.

Collagen and von Willebrand Factor may be considered as primary haemostatic agonists, whereas thrombin (generated by the coagulation cascade), ADP (released from platelet dense granules), and TXA_2 (synthesized and released by activated platelets) are secondary, but vitally important, stimulants. The role(s) of vasopressin, serotonin, IgG, and epinephrine receptors remain controversial, although each may play specific roles under specific hemorrhagic and/or thrombotic conditions.

Each agonist, to variable degrees, stimulates a range of common platelet responses.

Exocytosis of granular products: ADP, serotonin, calcium (each from dense granules), and fibrinogen (α granules) are important in the recruitment of platelets to the site of injury; growth factors (α granules) initiate vascular repair; and cytokines (α granules) and lysosomal enzymes (lysosomal granules) provide a link to the immune system.

Expression of granular membrane proteins: Adhesive proteins (e.g., GPIb, P-selectin, CD63, and several integrins - $\alpha IIb\beta_3$, $\alpha V\beta_3$, $\alpha 5\beta_1$, $\alpha 6\beta_1$, $\alpha 2\beta_1$) have been shown to be present on the membranes of intracellular granules and are expressed on the surface of activated platelets, thereby increasing the surface receptor density (11, 13, 16).

Eicosanoid formation: The arachidonic acid cascade is initiated, leading to TXA_2 synthesis, as outlined above. TXA_2 is a platelet agonist that plays a pro-aggregatory role, as described below.

Surface expression of adhesive receptors: There is a conformational change in the $\alpha IIb\beta_3$ integrin on the

Table 1. Receptors currently identified on human platelets

Agonist	Receptor
Von Willebrand factor	GPIb α
Collagen	$\alpha 2\beta_1$, GPVI
ADP	P2Y ₁ , P2Y ₁₂ , P2X ₁
Thromboxane A ₂	TP
Thrombin	PAR1, PAR4, GPIb
Platelet-activating factor	PAF receptor
Serotonin	5-HT ₂
Vasopressin	V ₁
IgG	Fc γ RIIA
Epinephrine	α_2
Fibrinogen	$\alpha IIb\beta_3$

platelet surface from an inactive to an active configuration, exposing a fibrinogen and von Willebrand Factor binding domain (RGD domain) on the α IIb β 3 integrin that facilitates inter-platelet binding (17).

Exposure of a pro-coagulant surface: The phospholipid composition of the external leaflet of activated platelets becomes phosphatidylserine-enriched and platelets shed phosphatidylserine-enriched microparticles. Both of these events provide surfaces that facilitate coagulation factor binding, activation, and thrombin generation (18).

The consequence of this range of platelet responses is the consolidation of the fragile platelet mono-layer into the hemostatic plug (15).

Atherosclerosis and arterial thrombosis

Atherosclerosis is the most frequent underlying cause of ischemic heart disease and cerebrovascular disease, the leading causes of morbidity and mortality in the developed world. The pathogenesis of atherosclerosis is multi-factorial involving both the deposition of lipids within the artery wall and the interaction of cellular responses characteristic of inflammatory disease (19, 20). The initial event in the formation of an atherosclerotic plaque is endothelial dysfunction caused by one or several of the following: elevated levels of low-density lipoproteins, hypertension, diabetes mellitus, increased free radicals, elevated homocysteine concentrations, or infectious agents such as chlamydia (21). The endothelial dysfunction manifests as increased adhesiveness for platelets and leukocytes and loss of anti-coagulant properties; these result from expression of adhesion molecules, chemokines, growth factors, and inflammatory mediators not present on normal endothelium (22 – 25). The inflammatory response stimulates the migration and proliferation of smooth muscle cells and the accumulation of monocyte-derived macrophages and T lymphocytes at the site of injury. Activation of these cells propagates the inflammatory response with further release of proteases, cytokines, and growth factors, and the development of the complex atherosclerotic lesion containing lipid and necrotic tissue covered by a fibrous cap (19 – 21).

Atherosclerosis itself is usually not fatal; the lethal consequences of acute myocardial infarction (MI) or ischemic stroke result from acute thrombosis superimposed on a chronic atherosclerotic plaque that has ruptured or eroded. The rupture of plaques is the most important underlying mechanism for the sudden progression of coronary vascular lesions and can occur because of factors intrinsic to the plaque such as cap thinning or extrinsic factors such as sudden increases

in blood pressure or heart rate. The ruptured plaque exposes thrombogenic subendothelial matrix proteins and collagen, triggering the cascade of platelet-mediated events, outlined above that lead to the formation of a platelet-rich clot and arterial vascular occlusion. Platelets adhere to collagen and von Willebrand Factor in the subendothelium and become activated by the thrombogenic surface or locally present agonists. The activated platelets bind adhesive proteins such as fibrinogen and aggregate, forming a platelet plug (26). They also release platelet agonists that activate and recruit more platelets to the growing thrombus and provide a catalytic surface for the assembly of coagulation factor zymogens and enzymes, leading to thrombin generation and fibrin formation. These platelet-rich thrombi are responsible for the acute occlusion of stenotic vessels and ischemic injury to heart and brain (27).

The central role played by platelets in acute arterial thrombosis has been demonstrated by a number of clinical observations. Ex vivo, enhanced responses to ADP and spontaneous platelet aggregation are more common in patients with both recent coronary events and subsequent events. These patients also have elevated levels of TXA₂ metabolites in their urine and β -thromboglobulin in their blood, markers of platelet activation. Increased numbers of circulating platelet aggregates are seen in patients with unstable angina and increased numbers of platelet microthrombi in patients with MI (28). An appreciation of the platelet's contribution to arterial thrombosis has made anti-platelet therapy a cornerstone of treatment and prevention for cardiovascular disease and stroke.

Inhibitors of PG-mediated platelet activation

Aspirin

Aspirin is the most widely used inhibitor of platelet function. It irreversibly inhibits PGHS-1, suppressing the synthesis of TXA₂. Platelet activation results in the liberation of arachidonic acid from the plasma membrane by PLA₂ and PLC. PGHS-1, an enzyme with two catalytic sites, metabolizes arachidonic acid to PGG₂ and then to PGH₂ (29 – 31). Thromboxane synthase then modifies PGH₂ to form the agonist TXA₂. TXA₂ is released by platelets and stimulates specific surface receptors, which activate PLC, release intracellular calcium, and ultimately generate more TXA₂, a positive feedback loop, enhancing aggregation and recruitment of platelets to the primary plug (32).

Aspirin irreversibly acetylates the hydroxyl group of serine 529, preventing arachidonic acid binding to the active site of the cyclooxygenase-1 (COX-1) component

of PGHS-1. Platelets cannot synthesize new protein and therefore the aspirin effect is maintained for the life span of the platelet (7–10 days); COX-1 activity is regenerated as new platelets are produced and released into the circulation (33–35). In contrast, other non-steroidal anti-inflammatory drugs (NSAIDs), such as ibuprofen and indomethacin, compete with arachidonate for binding to the active site of COX-1 and produce a reversible inhibition. Ibuprofen has recently been shown to interfere with COX-1 inhibition by aspirin when taken immediately before the aspirin, as measured by inhibition of serum TX levels. Ibuprofen binding to the catalytic site of COX-1 may prevent access of aspirin to its target serine residue. It may, therefore, be detrimental for those taking aspirin to decrease their cardiovascular risk to also take NSAIDs on a regular basis (36).

A single aspirin dose of 160 mg will completely block platelet COX-1; the same effect can be achieved with daily doses of 30–50 mg (29). The effective onset is rapid because aspirin can acetylate platelet COX-1 in the portal circulation.

COX-1 is expressed in many tissues in addition to platelets, including endothelial cells where the end product of the arachidonic acid pathway is prostacyclin (PGI₂), a potent vasodilator and platelet inhibitor. Aspirin inhibits PGI₂ synthesis, but unlike platelets, endothelial cells have the capacity to synthesize new COX-1, regenerating their ability to synthesize PGI₂. There has been concern that higher doses of aspirin inhibiting endothelial PGI₂ synthesis might be less effective in preventing cardiovascular events. Clinically, doses of aspirin between 30 and 1500 mg are equally efficacious suggesting that platelet inhibition is the more important effect (37). This may be because endothelial cells have a second, inducible COX isoform COX-2, not present in platelets, that can synthesize PGI₂ in the presence of aspirin. The COX-2-specific inhibitor rofecoxib was shown to be associated with an increased incidence of cardiovascular events compared to non-selective COX inhibitors (38, 39), perhaps as the result of the selective inhibition of COX-2-mediated production of PGI₂, enhancing the risk of vascular injury, in the presence of an uninhibited platelet TXA₂ production (40). These observations underline the complexity of choosing COX inhibitors for patients with underlying cardiovascular disease.

Aspirin has been evaluated in both primary and secondary prevention of vascular events. Aspirin has been shown to be effective in secondary prevention in patients with established atherosclerotic vascular disease. For example, the Second International Study of Infarct Survival (ISIS-2) (41) evaluated patients in the setting of acute MI and found a 25% reduction in

mortality at 5 weeks for patients who received aspirin starting within 24 h of acute MI, when compared to those who received a placebo. There was also a 50% reduction in non-fatal reinfarction and nonfatal stroke. The meta-analysis of 46 studies done by the Antiplatelet Trialists' Collaboration (42) showed a 25% reduction of the long-term risk of recurrent infarction, stroke, or death from vascular events following acute MI, and the benefit was observed over a wide range of aspirin doses (75–1500 mg/day). Similar benefits were shown for prevention of vascular events in patients with unstable angina and for the prevention of recurrent stroke. A low dose regimen of 75–150 mg daily is appropriate for the secondary prevention of coronary or cerebrovascular ischemic events; there appears to be no advantage to higher doses (43).

The benefits of aspirin for primary prevention of MI have been established, particularly for individuals with additional risk factors. The Antithrombotic Trialists' Collaboration's recently updated meta-analysis of 60 studies (44) demonstrated a reduced incidence of serious vascular events in specific high risk groups including patients with diabetes, stroke, peripheral arterial disease, and chronic stable angina. Anti-platelet therapy decreased the risk of non-fatal MI by one third, non-fatal stroke by one quarter and vascular mortality by one sixth. The results of the Physician's Health Study are similar, showing a 44% reduction in the incidence of first MI over a 5-year span in middle-aged men taking aspirin compared to those receiving placebo (45). Data is still insufficient to determine aspirin's efficacy in primary prevention of stroke or in preventing cardiovascular events in women.

The major side-effects of aspirin, both gastrointestinal irritation and hemorrhage, are dose-related, while its anti-thrombotic effects are not, suggesting the use of as low a dose of aspirin as is effective in the various vascular disorders (29).

Aspirin resistance

Despite the beneficial effects described above, aspirin is a relatively weak inhibitor of platelet function and blocks only TXA₂-mediated activation. It has no effect on platelet activation by agonists such as thrombin, which act independently of TXA₂, or on shear stress-induced activation. It inhibits platelet aggregation and secretion stimulated by weaker agonists, but has no effect on platelet adhesion. There is also a subset of patients who demonstrate clinical "aspirin resistance", that is, develop an ischemic vascular event while taking aspirin. Original studies by Mehta et al. (46) showed that up to 40% of patients demonstrated resistance based on an evaluation of platelet aggregation studies

or bleeding times done following a dose of aspirin. In some patients, this resistance could not be overcome by an increase in the dose. Gum et al. (47) found that 5% of stable cardiac patients were aspirin resistant based on optical platelet aggregation assays, and 9.5% of the same group showed resistance when tested using the Platelet Function Analyzer (PFA)-100®.

Demonstrated aspirin resistance does have clinical significance, with poorer outcomes for resistant patients when compared to aspirin responders. In a case-control study from the Heart Outcome Prevention Evaluation (HOPE), those patients on aspirin who had evidence of higher levels of urinary TX metabolites, suggesting incomplete inhibition of TXA₂ synthesis, had twice the risk of MI and 3.5 times the risk of cardiovascular death compared to those with low levels of TX metabolites (48).

The mechanism of aspirin resistance is probably multifactorial and includes factors such as smoking, concomitant use of NSAIDs (as described above), increased platelet turnover and inadequate aspirin dosing (49). Mechanisms intrinsic to the platelet that could contribute to aspirin resistance include the following: synthesis of TXA₂ by aspirin-resistant COX-2, polymorphisms in the COX-1 gene that alter the active site, conferring resistance to acetylation, or genetic polymorphisms affecting platelet integrins α IIB β 3 and α 2 β 1 that alter expression or lower the threshold for receptor activation (50).

Routine testing of cardiovascular patients for aspirin resistance is not yet a standard of practice, but the availability of point-of-care instruments such as the PFA-100®, which can demonstrate the effects of aspirin on primary hemostasis, could be used to screen for aspirin resistance (51).

TXA₂ inhibitors

Inhibitors of TX have the theoretical advantage of not affecting synthesis of PGI₂ and its platelet inhibitory effects. Clinical trials to date, however, have not demonstrated an advantage of these drugs over standard aspirin. For example, ridogrel, a combined TXA₂ synthase inhibitor and TX-receptor antagonist, was compared to aspirin in patients with acute MI receiving streptokinase thrombolysis (Ridogrel Versus Aspirin Patency Trial (RAPT)) (52). There was no difference in vessel patency evaluated by angiogram in patients receiving either adjuvant drug, either because ridogrel is a relatively weak antagonist or because the inhibition of PGI₂ by aspirin in this setting did not affect its clinical efficacy.

Dipyridamole

Dipyridamole has both antiplatelet and vasodilatory properties. It may have more than one site of action: the inhibition of nucleotide phosphodiesterase, the enzyme that degrades cyclic AMP, and the inhibition of adenosine uptake, preventing its stimulation of adenylyl cyclase, may both be important. The efficacy of dipyridamole has been debated but in the ESPS-2 trial of secondary prevention in over 6000 patients with prior stroke or TIA, the combination of low-dose aspirin and dipyridamole was more effective in preventing recurrent stroke than either drug alone (53).

The α IIB β 3 integrin as a target for anti-thrombotics

Ligand binding to glycoprotein IIb/IIIa (integrin α IIB β 3) is required for platelet aggregation and formation of a hemostatic plug or pathological thrombus. There is at least theoretical evidence that patients with Glanzmann thrombasthenia, who lack α IIB β 3 on the platelet surface, and as a result have a significant bleeding disorder, are protected from atherosclerosis and its complications (54). Platelet activation leads to a conformational change in α IIB β 3 increasing its affinity for soluble fibrinogen and other adhesive molecules containing the RGD motif. Binding of fibrinogen leads to α IIB β 3-mediated signaling that results in recruitment of a network of signaling molecules and reorganization of the cytoskeleton, irreversible changes that stabilize the clot. Because of its pivotal role in platelet aggregation, α IIB β 3 became the target for the development of antagonists that could be used in the setting of acute arterial vascular occlusion (55).

There are three types of the α IIB β 3 antagonists: monoclonal antibodies or fragments, cyclic peptides based on the RGD motif, and peptidomimetics. Murine monoclonal antibodies were first studied in the mid-1980s and found to block platelet aggregation *in vitro*, and thrombosis *in vivo*. The subsequent clinical trials of intravenous bolus dosing and infusions have demonstrated that these drugs can be beneficial in the context of acute coronary syndromes.

Intravenous α IIB β 3 integrin antagonists

Abciximab (ReoPro®), the Fab fragment of a humanized monoclonal antibody, c7E3, was developed to minimize immunogenicity and prevent binding to Fc receptors and complement fixation. It binds tightly to α IIB β 3 and also to the vitronectin receptor, α v β 3, present on platelets, vascular endothelial cells, and smooth muscle cells (56). At concentrations that produce >80% occupancy of α IIB β 3, platelet aggregation is

inhibited, the bleeding time is prolonged, and reocclusion of coronary arteries made patent by thrombolysis is reduced (57, 58). A bolus of abciximab is usually followed by an infusion to maintain >80% receptor occupancy, because of the slow but constant dissociation of abciximab from the platelet surface. Once discontinued, platelet function returns to normal over 72 h, although abciximab is present on platelets for up to 2 weeks following its administration, with some redistribution among circulating platelets (58, 59).

The clinical efficacy of abciximab in acute coronary syndromes has been tested in four clinical trials that addressed its potential role in angioplasty (EPIC, EPILOG), refractory unstable angina (CAPTURE), and coronary artery stenting (EPISTENT) (60–63). The evidence from these trials supports the efficacy of abciximab in reducing the complications associated with percutaneous coronary interventions (PCI) and improving long-term survival (64).

The toxicity profile of abciximab includes bleeding, as it produces a thrombasthenia-like platelet defect in patients simultaneously treated with heparin or thrombolytics. In addition, it induced thrombocytopenia in a small percentage of patients in the above mentioned studies: 0.4–1.6% had platelet counts $<50 \times 10^9/L$ (65), and an even smaller number had more profound decreases in platelet numbers (66). The cause of the thrombocytopenia is uncertain, although an immune response to the abciximab may be responsible in some patients in whom a detectable antibody to the human-murine chimeric protein has been found. However, the development of thrombocytopenia precedes the usual time course of a primary immune response. The alternate hypothesis is that abciximab causes conformational changes in $\alpha IIb\beta 3$ that are recognized by pre-existing antibodies in the plasma of some patients, causing antibody-mediated platelet clearance (67). The thrombocytopenia can usually be treated with platelet transfusion support until the abciximab is cleared. Other types of immune responses, such as hypersensitivity reactions upon retreatment, have not been seen (68).

The low molecular weight, nonbiologic inhibitors have an advantage over this antibody fragment of lack of immunogenicity and are either small peptides with a sequence based on the RGD motif or peptidomimetics with conformational similarity to this motif.

Eptifibatide (Integrilin®), a synthetic cyclic heptapeptide based on the KGD motif of the snake venom peptide barbourin, blocks fibrinogen binding to $\alpha IIb\beta 3$. It has the advantage over some naturally occurring RGD-containing snake venom disintegrins that are less specific for $\alpha IIb\beta 3$, and may inhibit ligand binding by other integrins. The cyclical conformation increases the

affinity of the peptide for its binding site (dissociation constant = 120 nM) (69). Intravenous infusion rapidly inhibits platelet aggregation, but is reversed within 2–4 h of discontinuing the drug. Two clinical trials evaluating eptifibatide in the setting of emergency coronary intervention (IMPACT-II) and unstable angina (PURSUIT) showed that it was safe and moderately effective in acute coronary syndromes if the infusion dosage was adequate to inhibit platelet aggregation (70, 71).

Non-peptide antagonists, designed to mimic the shape of RGD sequences have also been developed, including tirofiban (Aggrastat®), which binds specifically to $\alpha IIb\beta 3$ with a dissociation constant of 15 nM and inhibits platelet aggregation in platelet-rich plasma at concentrations of 30–66 nM (72). Following intravenous dosing, it has a plasma half-life of 1–2 h. Its safety and efficacy were studied in clinical trials evaluating its effect in patients undergoing angioplasty (RESTORE) or with unstable angina (PRISM, PRISM-PLUS) where it showed benefits in preventing early adverse events, but did not improve long-term outcomes (73–75).

The multicenter randomized clinical trials cited above, involving more than 100,000 patients in evaluation of the clinical efficacy of intravenous $\alpha IIb\beta 3$ antagonists, have shown that although these agents have a beneficial effect in very specific circumstances, such as preventing ischemic complications in patients undergoing PCI, their benefits in the medical management of acute coronary syndromes and MI are limited (55).

Oral $\alpha IIb\beta 3$ antagonists

In contrast to the evidence of benefit for intravenous $\alpha IIb\beta 3$ antagonists, data on the efficacy of oral $\alpha IIb\beta 3$ antagonists has been discouraging. Oral agents were of interest because of the possibility of extending the benefit seen in the acute coronary setting to long-term prevention of recurrent events. However, the results of five large Phase III trials with four different oral agents (EXCITE, OPUS, SYMPHONY, SYMPHONY II, BRAVO) failed to demonstrate a reduction in long-term ischemic events, despite increased bleeding in the treated groups (76–78). In addition, several of these studies showed excess mortality in the groups receiving $\alpha IIb\beta 3$ antagonists, regardless of dose, or the concurrent use of aspirin (79, 80).

What is the problem with $\alpha IIb\beta 3$ antagonists?

The reasons for the unexpectedly poor clinical results with the $\alpha IIb\beta 3$ antagonists are better understood now than when the trials produced these unexpected results.

These agents, both oral and parenteral, have a therapeutic window that is much narrower than originally suspected. The reasons for this are several, including inadequate platelet inhibition, partial agonist effects leading to platelet activation, and platelet-mediated inflammation.

The efficacy of α IIB β 3 antagonists is dependent on the level of platelet inhibition—this must be >80% for maximum effect during PCI. Failure to achieve this level of inhibition is associated with failure to protect from ischemic events. In the TARGET trial, abciximab was found to be more efficacious than tirofiban in the setting of PCI because of more complete inhibition by abciximab at the doses chosen (81). Maintenance of adequate levels of drug may be aggravated by the difficulty in *ex vivo* monitoring of platelet inhibition by aggregometry (82).

In the long-term use of oral agents, inadequate platelet inhibition over time may have been one of the reasons for failure. In the trials of intravenous agents outside the setting PCI, lower concentrations and longer infusions were used than in the earlier PCI trials. An example is the GUSTO IV trial, comparing 24- and 48-h infusions of abciximab following bolus dosing, where poorer outcomes were associated with longer infusions, and maximal platelet inhibition may not have been sustained during the later hours of the infusion (83).

The effect of incomplete inhibition is not simply a lack of efficacy; it may produce a paradoxical prothrombotic state, as the antagonists act as partial agonists, stimulating platelet activation and the release of inflammatory mediators (84). The α IIB β 3-receptor antagonists can stimulate outside-in signaling through the receptor, resulting in calcium flux, TXA₂ production, and granule release. These signals are weak, but in the absence of platelet inhibition, the partial agonist activity can be detected *ex vivo* in the expression of activation markers such as P-selectin and CD63 (as demonstrated in the OPUS-TIMI 16 trial) (85).

At high concentrations, the effect of α IIB β 3 antagonists is inhibitory on the inflammatory activity of platelets, reducing IL-1 β , blocking platelet-leukocyte adhesion, and inhibiting thrombin formation. However, at low levels, these agents enhance release of P-selectin, leading to platelet-leukocyte aggregates and increases in soluble CD40 ligand (86, 87). CD40 ligand binds to the CD40 receptor on monocytes, macrophages, and endothelial cells stimulating the release of cytokines and the expression of adhesion molecules, that is, an inflammatory response (88, 89).

Implications

The present recommendations for intravenous α IIB β 3-

receptor antagonists are limited to several specific situations. Abciximab is the preferred agent for PCI in patients also treated with heparin and aspirin (90). Tirofiban and eptifibatide are the agents of choice for medical management of acute coronary syndromes without instrumentation and are of most benefit in high-risk patients with diabetes mellitus or elevated levels of troponin (84). There is presently no role for prolonged infusions or the use of oral agents in the prevention of late events.

ADP receptors as targets for anti-thrombotics

Although the fundamental importance of ADP as a platelet stimulant *in vivo* has long been recognized, the receptors mediating ADP-induced platelet activation have, until relatively recently, been elusive. Molecular cloning techniques have, however, identified three distinct ADP receptors (P2Y₁, P2Y₁₂, P2X₁) on the surface of human platelets, each with distinct signaling pathways and functions (91–94). P2Y₁ and P2Y₁₂ are both serpentine, G-protein-linked receptors which are associated with G_q (stimulation of PLC β) and G_i (inhibition of adenylyl cyclase), respectively.

In platelets the engagement of P2Y₁ by ADP initiates the G_q/PLC β pathway. PLC hydrolyzes PIP₂ to yield IP₃, which releases stored calcium to elevate [Ca²⁺]_i and activates myosin light chain kinase, and DAG, which activates protein kinase C (PKC). DAG/PKC and IP₃/[Ca²⁺]_i are generally believed to be two of the key pathways mediating platelet response (7, 93, 95, 96). The P2Y₁ receptor is generally seen as a mediator of ADP-induced shape change and as a “trigger” which primes the α IIB β 3 integrin (93).

The P2Y₁₂ receptor is linked to G_i and thereby inhibits the activity of adenylyl cyclase and blocks the formation of cAMP (a major intracellular inhibitor of platelet function) (93). The signaling which occurs distal to the P2Y₁₂/G_i-mediated inhibition of adenylyl cyclase is unclear, although a PI₃-kinase-dependent, PKC-independent expression of binding domains on the α IIB β 3 integrin has been demonstrated (97). Stimulation of P2Y₁₂ amplifies the platelet response to ADP and is critical for full activation of the α IIB β 3 integrin, and thus is necessary for irreversible platelet aggregation, as well as granule exocytosis and the exposure of the phosphatidylserine enriched procoagulant surface (94, 98).

Little is known about the function of P2X₁ in ADP-induced platelet activation, at least partly due to the extreme sensitivity of this receptor to desensitization. However P2X₁ is a ligand-gated non-selective cation channel that appears to be responsible for Ca²⁺ entry in

response to ADP (99) and likely plays a role in ADP-induced shape change (94).

Ticlopidine and clopidogrel are thienopyridine derivatives which, although having no effect on agonist-induced platelet aggregation *in vitro*, have found clinical usefulness as anti-thrombotics (4, 5, 100–102). Both compounds are metabolized by hepatic cytochrome P450-1A (103) and, although structurally similar (clopidogrel has an additional carboxymethyl side chain), share no common metabolites (104). The active metabolites of clopidogrel (2-{1-[(1S)-1-(2-chlorophenyl)-2-methoxy-2-oxoethyl]-4-sulphonyl-3-piperidinyli-diene} acetic acid with an S configuration at C 7 and a Z configuration at C 3-C 16 double bond) (105, 106) and ticlopidine (to date, the active metabolite of ticlopidine is unknown) selectively and irreversibly bind to P2Y₁₂, thereby preventing ADP-induced activation of the α IIB β 3 integrin, fibrinogen binding, and platelet aggregation (4, 5, 101, 102). Interestingly, neither compound affects ADP-induced calcium influx or shape change, consistent with P2X₁, rather than P2Y₁₂, mediating these effects. Clopidogrel has largely superceded ticlopidine due to superior potency (approximately sixfold), more rapid onset, and lower incidence of limiting adverse effects, notably neutropenia and thrombotic thrombocytopenic purpura (101, 102).

A number of extensive clinical trials have confirmed a role for thienopyridine derivatives as anti-thrombotics. The landmark study was the clopidogrel versus aspirin in patients at risk of ischaemic events (CAPRIE) trial which demonstrated an 8.7% relative risk reduction in patients receiving clopidogrel for MI, ischemic stroke, or vascular death (107). If MI alone was taken as the outcome, there was a 19.2% relative risk reduction in patients receiving clopidogrel. In patients who had undergone cardiac surgery, the trial showed a 31.2% relative risk reduction for MI, ischemic stroke, vascular death, or re-hospitalisation (e.g., unstable angina, transient ischemic events) in the clopidogrel arm. Interestingly, there was no difference in the incidence of neutropenia or thrombocytopenia (107).

The Clopidogrel in Unstable Angina to Prevent Recurrent Ischemic Events (CURE) study compared the effects of aspirin with that of a clopidogrel/aspirin combination in patients with acute coronary syndromes (108). There was a 20% relative risk reduction for patients given the clopidogrel/aspirin combination, and although this was associated with an increased risk of bleeding, the incidence of severe hemorrhage was similar in the two arms.

The Stent Anticoagulation Restenosis Study (STARS), the Multicentre Aspirin and Ticlopidine Trial after Intracoronary Stenting Study (MATTIS), and the Intra-

coronary Stenting and Antithrombotic Regimen Study (ISAR) each demonstrated that there were significantly fewer restenoses in patients undergoing stent insertion given a ticlopidine/aspirin combination compared to aspirin alone (109–111). Subsequently, the Clopidogrel Aspirin Stent International Cooperative Study (CLASSICS) compared the effects of clopidogrel/aspirin combinations versus ticlopidine/aspirin combinations in patients undergoing coronary stenting (112). This study demonstrated that the clopidogrel/aspirin combination was at least as effective as the ticlopidine/aspirin combination in preventing post-stenting cardiovascular events; importantly, clopidogrel was superior with respect to adverse effects.

A number of additional clinical trials are underway to determine the usefulness of clopidogrel or clopidogrel combination therapies in a range of other situations, including acute ST-segment elevation in MI, high-risk transient ischemic attack or stroke, peripheral arterial bypass surgery, and heart failure (5).

In contrast to the thienopyridine derivatives which are pro-drugs, the ATP analogue AR-C69931MX (*N*⁶-(2-methyl-thioethyl)-2-(3,3,3-trifluoropropylthio)- β , γ -dichloromethylene ATP) is a competitive P2Y₁₂ antagonist that attenuates ADP-induced platelet aggregation *in vitro* (113). AR-C69931MX, which is not orally active, is a more effective inhibitor of platelet aggregation than clopidogrel. However in common with clopidogrel, but unlike aspirin or α IIB β 3 antagonists, AR-C69931MX also attenuates platelet-neutrophil and platelet monocyte interactions (114). Preliminary clinical trials and animal model studies have shown that AR-C69931MX prevents platelet-mediated thrombosis and maintains myocardial perfusion in acute coronary syndromes (115, 116). Other selective P2Y₁₂ antagonists, along with P2Y₁ and P2X₁ inhibitors, are at various stages of evaluation as anti-thrombotics (5).

Potential targets for anti-thrombotics

Although current anti-platelet therapy is dominated by cyclooxygenase inhibitors, α IIB β 3 blockers and ADP antagonists, a variety of other platelet receptors have been identified as potential targets.

Thrombin receptors

Thrombin is the most potent known platelet agonist (117). Platelets are known to express several thrombin receptors/binding sites on their surface, including GPIIb α and two members of the now well-characterised protease-activated receptor (PAR) family, PAR1 and PAR4 (117–121). GPIIb α is a high affinity thrombin-binding site on platelets. The role of GPIIb α in thrombin-

induced platelet activation is unclear, however its engagement may accelerate the hydrolysis, and action, of PAR1 (122), and may play an important role at low thrombin concentrations. The PARs are likely moderate affinity thrombin receptors on platelets. Thrombin binds to the PARs on the platelet surface and proteolytically removes a segment of terminal amino acid chain to generate a new extracellular domain, termed a tethered ligand, which subsequently auto-stimulates other regions of the receptor. Synthetic peptides corresponding to the terminal 6 amino acids of tethered ligands, SFLLRN (PAR1) and GYPGKF (PAR4), activate human platelets (117, 119, 120).

The thrombin receptors, particularly the PARs, are attractive targets for anti-thrombotic therapy; and indeed, selective antagonists have been synthesized including the PAR1 peptide-based antagonist RWJ-56110 (123) and the PAR4 inhibitor YD-3 (1-benzyl-3(ethoxycarbonylphenyl)-indazole) (124). Studies with these, and other antagonists, have shown that although inhibition of either receptor sub-type alone has minimal effect on thrombin-induced platelet activation *in vitro* (119), blockade of both PAR1 and PAR4 does attenuate platelet activation (119, 120). However, none of these antagonists, or antagonist combinations, have shown sufficient efficacy to warrant clinical evaluation.

Recently Covic and colleagues have reported an interesting, and potentially important, advance in the manipulation of thrombin receptor activation (125). Pepducins are membrane permeable, lipidated peptides that selectively adhere to intracellular domains of serpentine receptors, leading to either activation (pepducin agonists) or inhibition (pepducin antagonists) of the receptor. The pepducins are believed to act, likely at multiple sites, on the intracellular domain of the receptors to either activate (agonist) or attenuate (antagonist) the interaction of the receptor with the signal-transducing GTP-binding protein. In platelets, a PAR1 pepducin antagonist, P1pal-12, blocks PAR1-mediated intracellular signaling and aggregation, in the absence of any action on PAR4 (125). This suggests that there is a significant degree of selectivity in the pepducin activity. These observations, although at an early stage from a therapeutic perspective, represent an additional mechanism whereby platelet reactivity may be regulated in thrombotic conditions. Not only are the thrombin receptors, or other serpentine receptors (e.g., ADP receptors, TX receptors), targets, but any receptor associated with platelet activation and containing a unique intracellular signaling domain, such as the α IIB β 3 integrin, could be targeted (126).

The leptin receptor

Human platelets contain the long form (Ob-Rb) of the leptin receptor (127), a member of the class 1 cytokine receptor family (128). Although leptin does not itself cause platelet aggregation (127, 129), several (127, 129, 130), but not all (131), studies have shown that pre-incubation with leptin enhances ADP- and thrombin-induced aggregation, in a concentration-dependent fashion (127). These concentrations, however, are in excess of physiological levels (128), which may indicate Ob-Rb-independent effects of leptin (128).

Regardless, the effects of leptin on platelet aggregation have led to the suggestion that the thrombotic complications associated with obesity may result from enhanced platelet activation mediated by elevated circulating leptin levels (129, 132). However, it has recently been shown that the enhancing effects of leptin on ADP-induced aggregation is attenuated in overweight and obese individuals (130), consistent with Ob-Rb desensitization. Consequently it is unlikely that the effects of leptin on platelets accounts for obesity-related atherothrombotic conditions, although it remains theoretically possible that leptin receptor antagonists may have anti-platelet activity in conditions that are non-obesity-related. Indeed anti-Ob-Rb antibodies reverse the effects of leptin on ADP-induced aggregation (130), suggesting an Ob-Rb-mediated, rather than an independent effect of leptin on platelets. To date no selective antagonists of Ob-Rb have been reported.

The serotonin handling system

Selective serotonin reuptake inhibitors (SSRI) have represented a major advance in the management of psychiatric disorders (133). Drugs such as fluoxetine and paroxetine are well-tolerated, safe anti-depressants that have been widely used in conditions such as depression, anxiety, obsessive compulsive disorder, panic disorder, social phobia, and post-traumatic stress disorder (134). Although adverse effects of SSRI therapy, such as nausea, drowsiness, xerostomia, and headache, are well documented, an increased bleeding risk has also been reported (135–138). This bleeding, which is unique to SSRI anti-depressants, is both similar to (136), and enhanced by (138), NSAID use.

Platelets actively accumulate serotonin into dense granules (13, 139) and possess PLC β -linked serotonin receptors that are associated with platelet activation (139, 140). Studies on the effects of SSRIs on both platelet function and biochemical responses have been inconclusive. In many cases the likely pathological effects of various psychological conditions on platelet serotonin uptake/receptors have clouded the interpretation. Although there is general agreement that

SSRIs inhibit platelet serotonin accumulation (141–143), various SSRIs have been shown to inhibit platelet function in some (141, 142, 144–146), but not all (147), studies. Furthermore SSRIs have been reported to enhance (142) or decrease (148) serotonin receptor binding and increase both basal and stimulated changes in $[Ca^{2+}]_i$ (149).

Likewise the putative vascular protective effects of SSRIs are controversial. Two studies by Sauer and colleagues suggested that SSRI antidepressants, but not non-SSRI anti-depressants, reduced the incidence of MI (150, 151). In contrast Meier and colleagues saw no protective effects of SSRIs against a first MI (152), and Bak and colleagues reported that SSRIs did not decrease the risk of ischemic stroke or increase the risk of intracerebral hemorrhage (153). Consequently, it appears that the critical basic and clinical studies that would establish, or otherwise, the role of SSRIs as anti-thrombotics remain to be carried out.

Gas6

Gas6 (Growth Arrest-Specific gene 6) is a secreted protein, highly homologous to Protein S, which is present in, and released from, platelets (154), and which has been implicated as a potentially important component of the thrombo-inflammatory response (155). Gas6 is known to interact with three receptors, Axl (also known as UFO, ARK, Tyro7), Sky, and Mer, each of which is also found on platelets (156). The addition of Gas6 potentiates platelet aggregation in response to other agonists (157), and, critically from a potential anti-thrombotic point of view, inhibition of Gas6 binding, or the absence of any of its receptors, attenuates agonist-induced platelet activation (156, 157). The manipulation of Gas6 and/or its receptors is an interesting but untested approach to anti-thrombotic therapy.

CD40/CD40 ligand

The CD40/CD40 ligand interaction has been implicated in the pathogenesis of a variety of diseases including inflammation (158), Alzheimer's disease (159), atherosclerosis (158, 160), auto-immune disorders (161), and thrombosis (162). CD40 is a member of the TNF cytokine receptor super-family that has been shown to be present on the surface of a variety of cells including both vascular endothelial cells (163) and platelets (164, 165). CD40 ligand (CD154), a glycoprotein structurally related to TNF, exists in both membrane bound and soluble forms. Platelet activation leads to both the surface expression of membrane bound CD40 ligand (166) and the aspirin-sensitive secretion of the soluble CD40 ligand (167, 168); platelets may represent the major source of circulating soluble CD40

ligand.

The physiological/pathological function of platelet-associated CD40/CD40 ligand is not well characterized. Soluble CD40 ligand is elevated following cardiopulmonary bypass (169), during episodes of acute coronary syndrome (170, 171), and correlates with coronary artery lesions in Kawasaki disease (172). Consistent with a role for the CD40/CD40 ligand complex in thrombosis, soluble CD40 ligand interacts with expressed CD40 to release further soluble CD40 (164) and to stimulate platelet exocytosis and shape change (165). Interestingly conformational changes in the $\alpha IIb\beta 3$ integrin were also observed although of a magnitude which did not support platelet aggregation (165). In contrast, CD40 ligand does interact with a RGDS-binding domain on the $\alpha IIb\beta 3$ integrin and serves to stabilize thrombi formed under high shear conditions such as that occurring in arteries (173). Of particular interest, platelet-bound CD40 ligand, expressed following activation, will interact with endothelial CD40 to stimulate pro-inflammatory changes (166), which may provide a crucial link between inflammation and thrombosis. Therapeutically, intravenous immunoglobulin significantly decreased CD40 ligand levels in Kawasaki disease leading to decreased vascular damage (172). These recent observations suggest that the CD40/CD40 ligand system is a potential target for anti-thrombotic therapy.

Concluding remarks

The role of anti-platelet therapy in certain cardiovascular disorders is now unequivocal. Individual anti-platelet agents, such as aspirin, the intravenous $\alpha IIb\beta 3$ integrin antagonists and the thienopyridine derivatives have become standard in both the prevention and treatment of conditions such as MI, stroke, and unstable angina. As more is learned of platelet structure and function, a host of new targets for anti-platelet therapy have presented themselves, including the thrombin receptor, the serotonin handling system, and the leptin receptor. However the recent experience with oral $\alpha IIb\beta 3$ integrin antagonists and thromboxane receptor antagonists cautions that pharmacological modulation of platelet interactions with other components of the vascular system can have unforeseen consequences.

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