Therapeutic approaches in arterial thrombosis


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Summary. The current standard of care for the treatment of arterial thrombosis includes anticoagulants and three classes of antiplatelet agents – aspirin, thienopyridines and glycoprotein IIb-IIIa antagonists. Although these drugs have had a significant impact on morbidity and mortality in several patient populations, up to 15% of the high risk patients with acute coronary syndrome continue to suffer from ischemic events. This problem may occur, in part, because the platelets in many patients are non-responsive to aspirin and clopidogrel. Murine models now indicate that platelets are not only responsible for arterial occlusion, they are also involved in the progression of atherosclerotic disease. New opportunities have emerged identifying potential targets and strategies for drug discovery suited to address these deficiencies by more effectively modulating platelet adhesion, thrombus growth, thrombus stability and the pro-inflammatory activity of platelets. In addition, a growing need has emerged for the development of bedside devices capable of bringing personalized medicine to patients being treated with antithrombotic therapies in order to measure the pharmacodynamic activities of these new therapies, to assess the activities achieved by combined antithrombotic therapy, and to identify patients that fail to respond.

Keywords: anticoagulant, antiplatelet, atherosclerosis, platelet, platelet monitoring, thrombosis.

Introduction

Arterial thrombosis is the result of sequential events involving platelet adhesion, activation and subsequent aggregation that can lead to vascular occlusion, perhaps the primary pathological complication of advanced atherosclerotic lesions. Recent advances in the field of thrombosis suggest that the second pathological consequence of platelet adhesion and activation may be as consequential as the immediate ischemia induced by arterial thrombosis as platelets are a primary source of several inflammatory proteins known to be involved in the progression of atherosclerotic disease including RANTES, sCD40L, PDGF and transforming growth factor-β (TGF-β). These considerations suggest that therapeutic targeting of platelets has two objectives: first, prevention of vessel occlusion; second, inhibition of the platelet contribution to lesion progression.

The pharmaceutical industry has made important inroads into the development of drugs for the treatment of the thrombotic complications of atherosclerosis. In one example, the occlusive, ischemic consequences of acute myocardial infarction (MI) have been addressed, by thrombolytics to lyse the thrombus, and more recently by interventional strategies to mechanically remove or dislodge the thrombus and to maintain artery patency with stents coated with agents such as rapamycin or paclitaxel to reduce the incidence of restenosis. In yet another example, antagonists against platelet receptors such as glycoprotein (GP) IIb-IIIa and P2Y12 have been developed, joining aspirin, a Cox-1 inhibitor as the primary antithrombotic drugs. However, despite these advances in antithrombotic therapies and the widespread use of statins to reduce cholesterol and CRP levels, the incidence of atherosclerosis continues to rise, as do the ischemic consequences of atherosclerosis including MI and stroke. An added complication is type 2 diabetes which is an independent risk factor for cardiovascular disease (CVD). A recent analysis of the Framingham Heart Study showed that even though management of risk factors such as blood pressure and cholesterol has improved significantly in the total patient population, the presence of diabetes significantly reduced the overall benefits [1]. While it may be possible to address these pathologies by more aggressive health management, and by more optimal application of existing therapies, clearly, truly effective treatment of the thrombotic consequences of atherosclerosis requires not only the development of drugs to be used as primary care on a chronic basis to prevent thrombosis and its ischemic complications but also to block the contribution of platelet-induced inflammation in the progression of atherosclerotic disease. Reviewed below is the mechanism of action, clinical successes and limitations of the four drug classes currently used to prevent arterial thrombosis; aspirin, P2Y12 inhibitors, GP IIb-IIIa antagonists and anticoagulants. Therapeutic opportunities afforded by our current understanding of the mechanisms of arterial thrombosis and the inflammatory activity of platelets are discussed. Finally, recognizing that individuals vary in response to various drugs and that combination antithrombotic therapies has become commonplace, we will highlight the need for improved pharmacodynamic assessment of platelet function.
Current strategies

Current therapeutic strategies for the treatment of arterial thrombosis are based on the well-known receptor systems summarized in Fig. 1. In this simplified diagram, collagen and/or thrombin are designated as the primary platelet agonists. While either agonist is capable of activating platelets, including the activation of the receptor function of GP IIb-IIIa for the binding of fibrinogen and von Willebrand factor (VWF) to initiate platelet aggregation, stable aggregation of platelets is augmented by two autocrine factors generated upon platelet stimulation: ADP, released from platelet dense bodies, and TXA₂, generated by the sequential actions of Cox-1 and thromboxane synthase on the arachidonic acid released from membrane phospholipids. Additional aggregation-dependent secondary mediators include sCD40L and Gas6 plus aggregation-induced tyrosine phosphorylation of GP IIb-IIIa and activation of secondary aggregation receptors such as SLAM, CD84, Eph kinase and the Gas-6 receptors. Even though the signaling reactions induced by the receptor systems for the platelet stimuli summarized in Fig. 1 are diverse, including those coupled by Gᵦ₁ (PAR-1 and TP), Gᵦ (P₂Y₁₂), Syk (GP VI), She and talin (GP IIb-IIIa), drugs that target these receptor systems have been designed either to specifically inhibit the receptors themselves [e.g. GP IIb-IIIa antagonists (eptifibatide, abciximab, tirofiban) and P₂Y₁₂ inhibitors (clopidogrel, ticlopidine)], to block the generation of the agonists [e.g. the Cox-1 inhibitor aspirin and Factor Xa (FXa) inhibitors [low molecular weight (LMW) heparins]], or to antagonize the agonist itself (e.g. thrombin inhibitors [standard heparin, direct thrombin inhibitors]).

Aspirin

The clinical successes achieved by the current therapies to treat arterial thrombosis have been remarkable. Aspirin was the first and continues to be the most widely used of these drugs. The trend toward the widespread use of this drug to block arterial thrombosis was first indicated by the findings of the ISIS-2 trial which demonstrated that aspirin reduced mortality from acute MI to a rate that is similar to that of the thrombolytic agent, streptokinase [2]. The data from multiple trials summarized by the Antiplatelets Trialist’s Collaboration found a 25% relative risk reduction by aspirin of vascular death, MI or stroke, vs. placebo [3] which led to the widespread adoption of aspirin as standard therapy for primary and secondary prevention of arterial ischemia. This collaboration also reviewed the clinical trials using aspirin to show that low-dose aspirin (75–150 mg daily) is effective for long-term use [4]. While the half-life of aspirin in humans is relatively short (~20 min), its effect persists for the lifetime of an affected platelet in circulation as the drug acetylates Cox-1 at serine-529, located at the active site of the enzyme. Attempts have been made to develop additional drugs that target the thromboxane pathway in platelets including a variety of thromboxane receptor (TP) antagonists, thromboxane Α₂ synthase inhibitors, or compounds that combined both functions [5]. Although some of these agents had potent antithrombotic effects in experimental models and preclinical studies, and offered the advantage of inhibiting the TP stimulating activity of prostanoid metabolites in addition to TXA₂ (e.g. isoprostanes, PGH₂), they are not currently used to block arterial ischemia as most were not evaluated in clinically relevant phase III trials [6].

Thienopyridines

The second most widely used of the antiplatelet drugs for chronic use are thienopyridines targeting P₂Y₁₂. This class of drugs, which includes clopidogrel, and its predecessor ticlopidine, act via irreversible inhibition of the platelet P₂Y₁₂ receptor. Both are prodrugs, requiring hepatic metabolism by
cytochrome P450 isoform 3A4 in order to generate the active metabolite, a transient intermediate which covalently modifies and inactivates the receptor. Ticlopidine has been shown to be efficacious in conditions such as claudication, unstable angina, and cerebrovascular disease [7]. However, the incidence of neutropenia associated with ticlopidine led to the development of a second-generation thienopyridine, clopidogrel, with increased potency and fewer side-effects. In the CAPRIE trial [8], clopidogrel was shown to be more efficacious than aspirin, particularly in high-risk patients (diabetics and those with a history of prior revascularization). Subsequently, the CURE study [9] demonstrated that patients with unstable angina or non-ST segment elevation MI received a 20% relative risk reduction if they were randomized to clopidogrel plus aspirin vs. placebo plus aspirin, and the PCI-CURE substudy [10] showed that this benefit extended to patients undergoing percutaneous intervention (PCI). The slow onset of action of thienopyridines, due to their metabolism requirement, has necessitated the administration of a large loading dose (300 mg) prior to acute procedures, such as PCI, as demonstrated in the CREDO trial, where the maximum benefit of clopidogrel administered with aspirin required a loading dose given at least 6 h prior to the procedure. This study also demonstrated a significant 27% reduction in death, MI and stroke from 1-year administration of clopidogrel plus aspirin following PCI, compared to 1-month dosing [11].

**GP IIb-IIIa antagonists**

The GP IIb-IIIa antagonists are designed to bind to the integrin on unstimulated platelets and on platelets after stimulation. GP IIb-IIIa is an attractive antiplatelet target as it is (i) on the ‘final common pathway’ mediating platelet aggregation irrespective of the agonist used to induce platelet activation, (ii) platelet-specific, and (iii) responsible for a variety of aggregation-dependent platelet functions including those in coagulation, inflammation, fibrinolysis and vascular cell proliferation. Three GP IIb-IIIa antagonists have been developed: integrilin, a cyclic heptapeptide modeled after the active site of the disintegrin found in the southeast pigmy rattle snake; abciximab, a Fab fragment of a mouse/human chimeric antibody against GP IIb-IIIa; and tirofiban, a synthetic inhibitor of GP IIb-IIIa. All were designed to be infusable i.v. drugs and are therefore only administered to patients in acute settings who have a high risk of experiencing an ischemic event such as those undergoing PCI (with or without stent placement) or those with symptoms resulting from acute coronary syndrome (ACS) [12]. Use of these drugs has shown a remarkable reduction in death and MI for these indications [13–15].

**Anticoagulants**

ACS patients, whether undergoing an invasive revascularization procedure or not, are treated with aspirin and antithrombin therapy in the form of unfractionated or LMW heparins. Although unfractionated heparin is effective in reducing clinical events, a narrow therapeutic index makes it a less than optimal antithrombin for this class of patients. Like their parent anticoagulant, i.e. standard heparin, LMW heparins are indirect antithrombins and utilize antithrombin III to mediate inhibition of thrombin and FXa. As LMW heparins have more predictable pharmacokinetics than standard heparin, they are used in a fixed dose manner. Early trials of the LMW heparin enoxaparin in unstable angina and non-Q wave MI patients demonstrated improved efficacy over standard heparin and the drug has emerged as the most commonly used LMW heparin [16,17]. Fondaparinux, a synthetic pentasaccharide, also utilizes the antithrombin III binding region of heparin and has been found to be an appropriate anticoagulant for prevention of deep vein thrombosis in orthopedic surgery [18]. Unlike enoxaparin, which inhibits both thrombin and FXa, fondaparinux acts only as an indirect FXa inhibitor. Venous thromboembolism prevention trials showed that fondaparinux has a superior efficacy profile to its comparator enoxaparin. Ongoing trials of fondaparinux in ACS patients will show if the concept of attaining superior efficacy by inhibition of FXa alone (vs. the combination of FXa and thrombin) can be achieved in arterial settings.

**Combination antithrombotic therapy**

Arterial thrombosis developed at sites of spontaneously or mechanically disrupted atherosclerotic plaque is triggered by a multitude of highly thrombogenic materials (i.e. fibrillar collagen and tissue factor). It is the result of complex interrelations between coagulation and platelets orchestrated by local rheological conditions. An emerging strategy in the treatment of arterial thrombosis came with the realization that combinations of antithrombotics provide greater therapeutic benefit than are provided by drugs used singly. Accordingly, the combination aspirin-plus-clopidogrel is rapidly becoming the new standard of care for the management of patients with non-ST segment elevation ACS and in patients undergoing a PCI. In support of this trend, the CURE study demonstrated that aspirin-plus-clopidogrel caused a 20% relative risk reduction of vascular death, MI, and stroke compared with aspirin-plus-placebo [9]. The dual antiplatelet therapy (aspirin-plus-clopidogrel) was also more effective and safer than a combination aspirin-plus-warfarin in coronary artery stenting [19,20]. The remarkable efficacy of the dual anti-platelet therapy has prompted the initiation of several clinical trials in indications as diverse as atrial fibrillation, peripheral arterial disease, peripheral arterial bypass surgery, secondary and high-risk primary prevention, acute ST-segment elevation MI and heart failure [21]. Finally, although anticoagulants were routinely used in the development of antiplatelet agents, analysis of these data shows that these combinations often provided a clinical benefit that was greater than anticipated. We and others have used thrombosis models to evaluate synergisms between various pathways. Because TXA₂ and ADP activate different pathways, it was anticipated that combinations of inhibitors of the two pathways would confer a
greater protection against thrombotic events. However, rather than an additive effect, the two drugs used together were synergistic [22,23]. A potent synergism between clopidogrel and antiaggregants [using either a direct thrombin inhibitor (Bivalirudin), a FXa inhibitor or a synthetic LMW heparin] has also been observed (Fig. 2) [23,24]. While these results may have a simple explanation, resulting from the inhibition of fibrin formation by the antiaggregant, wherein fibrin contributes to thrombus stability under arterial shear rate [25], the emerging data indicate that the anti-thrombotic synergisms may originate from the complementation of the signaling pathways in platelets. Activation of the receptor function of GP IIb-IIIa is optimal when engagement of G12/13 or Gαs signaling pathways is combined with Gαi stimulation [26,27]. This combination occurs when either TXA2 (TP) receptor (G12/13, Gαi), protease-activated receptor-1 (PAR-1) (G12/13, Gαi), and also potentially P2Y1 (Gαi) [28] is allowed to synergize with that of P2Y12 (Gαi). Note that this model might also explain the synergism between aspirin and clopidogrel. A different explanation may stem from the inhibition of the different PI-3 kinase enzymes present in platelets. For example, P2Y12 engagement by ADP stimulates PI-3 kinase-γ whereas the engagement of either FCγRIIA/GPVI, PAR-1, TP, or GP IIb-IIIa lead to PI-3 kinase α or β-activation. This could indicate that any combination therapy that would block PI-3 kinase α, β or γ would confer a strong antithrombotic efficacy.

Limitations of current antithrombotics

Even with the remarkable successes that have been achieved with currently available antithrombotics in the prevention of arterial thrombosis, limitations of this class of drugs do exist. It is valuable, therefore, to consider additional strategies currently available to design new drugs that address these limitations. That there is room for improvement is readily apparent from analysis of current trials. For example in SYNERGY, a trial with more than 10000 high risk non-ST segment elevation ACS patients treated with heparin or LMW heparin, aspirin, and, as determined by the physician, clopidogrel and/or GP IIb-IIIa antagonists, approximately 15% of all patients still experienced death or non-fatal MI within 30 days of treatment [29]. Except for aspirin, perhaps the biggest limitation of these drugs is that the dose used is less than optimal for the treatment of thrombosis. The drugs were typically titrated in preclinical development studies to arrive at a dose that had a significant inhibition of thrombosis without undue bleeding. While higher doses of the drugs had better antithrombotic activity, this always created a bleeding risk. Although the dose selected required some refinement in subsequent clinical studies, this same strategy was employed.

Aspirin non-responsiveness

While aspirin is used at a dose (e.g. 70-325 mg day−1) that yields near 100% acetylation of Cox-1 in most individuals, it has been recognized for several years that individuals treated with aspirin still experience thrombotic events. The interpretation of this observation has been controversial. One could argue that aspirin is a comparatively ‘weak’ inhibitor in that it only blocks the production of TXA2, an autocrine factor that supplements the activities of the primary platelet agonists. Indeed, aggregation reactions using platelets from individuals taking aspirin appear normal when high concentrations of primary agonists are used. However, it has become clear from recent pharmacodynamic and biochemical studies that platelet responses normally blocked by inhibition of Cox-1 (the target of aspirin in platelets) are still present in some individuals, an observation that has led to the concept of ‘aspirin resistance’ or ‘aspirin non-responders’ [30-32]. Studies of platelet aggregation in aspirin-treated CVD patients, both by traditional ADP and arachidonic acid induced aggregation studies and by platelet function analyzers such as PFA100, show that 5–10% of the individuals can be classified as aspirin non-responders [31]. Additional studies demonstrated a threefold higher risk of major cardiovascular adverse events in the same patient population [30]. As the incidence of aspirin non-responders had been shown to be higher in patients who undergo coronary artery bypass graft (CABG), a recent study evaluated the functional and biochemical responses to aspirin on subsequent days following a CABG procedure in a small number of patients [33]. The study demonstrated that platelets from these patients after CABG did not completely respond to aspirin in vitro, and that while Cox-1 levels in platelets remained constant 10 days following the procedure, there was a pronounced increase in the level of Cox-2, which is 170-fold less sensitive to aspirin inhibition, especially at 5 days post-procedure. This may be reflective of the increased platelet turnover following cardiopulmonary bypass, and the increased level of Cox-2 could generate critical amounts of TXA2, in spite of aspirin treatment, providing a possible explanation for aspirin non-responsiveness. Other mechanisms proposed as contributing factors to aspirin non-responsiveness include use of NSAIDs, which block acetylation by aspirin [34] and polymorphisms of platelet genes (Cox-1 or GP IIb-IIIa) [35], and non-compliance. However, the answer may be totally unexpected such as the activation of a deacetylase. Clearly, further
studies are needed to better define the underlying mechanisms of this phenomenon. Aspirin use is also contraindicated in a significant population of patients, i.e. those with gastrointestinal bleeding and those with aspirin-induced asthma.

**Clopidogrel non-responsiveness**

The primary limitation of clopidogrel is that this drug demonstrates weak and somewhat variable inhibition of P2Y12 [36]. Following a 600-mg loading dose of clopidogrel, the extent of inhibition of ADP-induced aggregation (5 μM ADP) varied from 33% to 78% in healthy individuals, at 6 h post-dosing [37]. This effect is further exaggerated in patients undergoing PCI/stent placement [38,39]. The antithrombotic effect of clopidogrel is likely to be dependent on a number of factors including but not limited to variations in P450s, polymorphisms of the P2Y12 receptor and receptor signaling pathways. Measurements of platelet aggregation and markers of platelet activation (GP IIb-IIIa and P-selectin detection by specific antibodies) show that clopidogrel resistance is detected in 31% of the patients on day 5 and 15% of the patients on day 30 of the treatment regimen [40]. A prospective study of PCI patients with non-ST segment elevation MI showed that up to 25% of the patients were resistant to clopidogrel [41]. When the patients were stratified into quartiles based on resistance to ADP-induced platelet aggregation, the most resistant patients had a 40% adverse event rate during a 6-month follow-up period so they are obviously being denied adequate protection based on inhibition of P2Y12. It has also been reported that the antiplatelet activity of clopidogrel is blocked in patients treated with a widely used cholesterol lowering medication (atorvastatin) which is undoubtedly linked to the metabolism requirement for efficacy [42]. A second limitation of these drugs stems from their irreversible mechanism of action, which inactivates the P2Y12 receptor for the lifetime of the platelet. While this is not a particular problem with aspirin, which is also irreversible, this feature has led to limited use of clopidogrel before PCI in patients who are at increased risk of undergoing CABG procedures, as the risk for bleeding following clopidogrel treatment requires postponement of the procedure for 5–7 days, or transfusion of large numbers of platelets during the procedure.

**Limitations of anticoagulants – i.v.**

Each anticoagulant has evolved unique issues. Replacement of unfractionated heparin has had mixed success. Although the current ACC/AHA guidelines (2002) prefer enoxaparin over unfractionated heparin, recent data do not support this preference. In the SYNERGY trial, enoxaparin was found not to be superior to unfractionated heparin [29]. In A to Z, for patients on a GP IIb-IIIa antagonist (tirofiban) and aspirin, enoxaparin was found to be non-inferior to standard heparin [43]. LMW heparins also have a narrow safety window. For example, in unstable angina patients treated with enoxaparin, a 25% dose increase in therapeutic level (1.25 mg kg\(^{-1}\)) produces an unacceptable number of bleeding events [44]. Direct thrombin inhibitors such as angiomax have also been studied as replacements for unfractionated heparin. In the REPLACE 2 trial of PCI, angiomax and provisional GP IIb-IIIa inhibitor compared favorably to heparin plus GP IIb-IIIa [45]. The primary end point of the trial combined efficacy and safety parameters and the angiomax arm of the trial was statistically not inferior to the heparin arm. However, the benefit related to reduction of bleeding with the use of angiomax is questionable. In REPLACE 2, the control group was likely to have been over anticoagulated as the observed ACT values in the heparin arm were higher than the recommended ACT range (200–300 s) for use of heparin in conjunction with GP IIb-IIIa. As angiomax is substantially more expensive than standard heparin, economic considerations also contribute to its limited use in PCI patients.

**Limitations of anticoagulants – oral**

Warfarin is the only anticoagulant in chronic use. While the drug provides tremendous benefit to affected individuals, its anticoagulant response is influenced by a variety of factors such that >50% of patients are usually outside of the therapeutic range. Due to the large variability in the anticoagulant effect of warfarin and its narrow therapeutic index, a large unmet clinical need exists for an anticoagulant with predictable fixed-dose usage. The need for a warfarin substitute has led to numerous drug development projects that have focused on inhibitors of coagulation proteases that specifically inactivate the protease active site. Ximelagatran, an oral thrombin inhibitor, was the first to show that the strategy of direct coagulation protease inhibition does translate into effective anticoagulation and leads to antithrombotic activity in deep vein thrombosis and atrial fibrillation patients [46]. Two studies suggest that ximelagatran is at least as effective as warfarin in preventing stroke in high-risk patients with atrial fibrillation [47]. The studies also showed that there are incidences of increase in liver enzymes which would require surveillance for potential liver toxicity in future patients. Unfortunately, safety problems of ximelagatran related to serious liver toxicity has led the FDA to recommend against approval of this thrombin inhibitor. While several new experimental agents with the potential to be an effective and low variability anticoagulant have been evaluated in clinical trials, none of these are available for therapeutic use, so the search for a warfarin replacement remains a work in progress.

**PAR-1**

While drugs that inhibit thrombin or prevent its formation are a mainstay in the armamentarium used for the treatment of arterial thrombosis, as of this writing, no efficacy trials have been performed to determine whether antagonists of PAR-1, the thrombin receptor on platelets, could provide a therapeutic benefit. Potent and selective PAR-1 antagonists capable of inhibition of thrombin-induced platelet aggregation have been
reported in the literature. Peptide mimetic antagonists such as RWJ-58259 are effective in models of thrombosis and vascular injury and could have potential as therapies for treating thrombosis and restenosis [48]. An oral PAR-1 antagonist, E-5555, is being developed as a drug candidate for ACS but definitive clinical efficacy trials have not been reported [49].

**Future directions for antithrombotic drug development**

New antithrombotics are required not only to overcome the limitations of the current drugs to better manage arterial ischemia, but also to address the inflammatory activities of platelets which contribute to progression of atherosclerotic disease. The successes and limitations of current therapies coupled with the advances made in our understanding of platelet biology are instructive in the design of new drugs to more effectively regulate validated targets, in the identification of new targets that may safely provide increased benefit and in the development of the proper combination of antithrombotics for the various arterial ischemic indications.

**Agonist receptors**

While platelets are activated by numerous agonists acting on multiple receptors, the only validated agonist receptor for drug discovery is P2Y12. The requirements for improvements over clopidogrel are clear – more potent inhibition of P2Y12; less variability of inhibition between different patients; no requirement for metabolism resulting in less delay in onset to action; and quicker recovery of platelet function following discontinuation of use. While these requirements can most likely be best achieved by an orally available reversible P2Y12 antagonist, preclinical data indicate three promising candidates in development with different properties. One is cangrelor (AR-C69931MX), a nucleotide, intravenous compound that reversibly antagonizes P2Y12 [50]. AZD-6140, an orally available direct-acting P2Y12 antagonist [51], is presently being evaluated in phase II clinical trials. Prasugrel (CS-747), a thienopyridine prodrug similar to clopidogrel which is more rapidly converted outside-in signaling by tyrosine phosphorylation of GP IIIa, a reaction which is defective in the platelets from the DiYF mouse [63]. While inhibition of primary platelet aggregation and this secondary aggregation mechanism are both inhibited by GP IIb-IIIa antagonists, a potential drug target is the metalloproteinase responsible for CD40L cleavage. Another protein released upon platelet activation that functions to consolidate platelet thrombi is Gas6. Gene targeting of Gas6 also demonstrates a thrombosis phenotype [64]. Gas6 binds to three receptors on platelets, Tyro3, Axl and Mer, but genetic targeting of any one unexpectedly inhibits Gas6-induced platelet stimulation. However, as Gas6 also induces tyrosine phosphorylation of GP IIIa, apparently by a mechanism independent of binding to the integrin, it has been proposed that Gas-6 signaling could be therapeutically regulated through inhibition of Gas6-GP IIb-IIIa cross-talk [65].

**Secondary aggregation receptors**

While the initial interaction of platelets during thrombosis is dependent upon GP IIb-IIIa, it has become apparent that signaling reactions initiated by platelet–platelet contact are required for thrombus stability. Several mediators of aggregation-induced signals have been identified. One is GP IIb-IIIa itself which becomes tyrosine phosphorylated and also associates with numerous signaling and cytoskeletal proteins following platelet aggregation. The importance of the ‘outside-in’ signaling in the enhancement of platelet aggregation was demonstrated by the generation of knock-in mice where tyrosine residues Y747 and Y759 were mutated to phenylalanine [60]. The so-called DiYF mice displayed selective impairment of outside-in signaling resulting in the formation of unstable aggregates. In addition, as shown in Fig. 2, ex vivo perfusion chamber experiments on type III collagen have shown that the DiYF mouse has defective thrombosis. Another protein involved in secondary platelet aggregation is CD40L, a tumor necrosis factor family member mainly expressed on activated T cells and platelets [see 61]. CD40L is cryptic in unstimulated platelets, but rapidly becomes exposed on the platelet surface after stimulation where it is subsequently cleaved, producing a soluble hydrolytic product termed sCD40L [61]. We have shown that mice lacking CD40L have a thrombosis phenotype and that normal thrombosis is regained upon infusion of sCD40L [62]. Interestingly, sCD40L, in addition to being a ligand for CD40, is also a ligand for GP IIb-IIIa, a reaction that depends upon its KGD sequence, a known GP IIb-IIIa binding motif. sCD40L also triggers outside-in signaling by tyrosine phosphorylation of GP IIIa, a reaction which is defective in the platelets from the DiYF mouse [63]. While inhibition of primary platelet aggregation and this secondary aggregation mechanism are both inhibited by GP IIb-IIIa antagonists, a potential drug target is the metalloproteinase responsible for CD40L cleavage. Another protein released upon platelet activation that functions to consolidate platelet thrombi is Gas6. Gene targeting of Gas6 also demonstrates a thrombosis phenotype [64]. Gas6 binds to three receptors on platelets, Tyro3, Axl and Mer, but genetic targeting of any one unexpectedly inhibits Gas6-induced platelet stimulation. However, as Gas6 also induces tyrosine phosphorylation of GP IIIa, apparently by a mechanism independent of binding to the integrin, it has been proposed that Gas-6 signaling could be therapeutically regulated through inhibition of Gas6-GP IIb-IIIa cross-talk [65].

Platelet–platelet contacts induce the activation of additional signaling mechanisms which are involved in aggregate stability. One involves Eph kinases and ephrins, specifically EphA4 and ephrinB1, which through receptor ligand interactions on the platelet surface enhance the binding of GP IIb-IIIa to immobilized fibrinogen in the presence of physiological agonists [66]. Recent work from our laboratory using both
oligonucleotide-based microarray analyses and mass spectrometric proteomics has identified two additional receptor families that are involved. One involves two members of the SLAM family of adhesion receptors, SLAM and CD84: the other involves a novel protein termed PEAR1 (N. Nanda, M. Hart and D.R. Phillips, unpublished data). All proteins are exposed on the surfaces of unstimulated platelets and signal secondary to GP Ib-IIIa-mediated platelet–platelet contacts by becoming tyrosine phosphorylated. Therapeutic targeting of one or more of these secondary aggregation receptor systems in platelets is an attractive possibility as they appear to have a greater effect on thrombosis than they do on hemostasis.

Adhesion receptors

Several platelet adhesion receptors have been identified but we will focus on GPVI and GPIb\(\alpha\), the adhesion receptors that are not only involved in the adhesion of platelets to the highly thrombogenic fibrillar collagens in the vessel wall exposed following vascular injury but also contribute to platelet activation. Both receptors are attractive drug discovery targets as both are platelet specific. Under the high shear rates encountered in coronary and carotid arteries, the binding of VWF to the collagen surface triggers a transient interaction with GPIb\(\alpha\) that allows for a more stable interaction of the platelet with the collagen surface triggers a transient interaction with GPIb\(\alpha\) that allows for a more stable interaction of the platelet with the collagen surface (via at least two collagen receptors, integrin \(\alpha_\beta_1\) and GPVI. Recent findings indicate that GPIb\(\alpha\) and \(\alpha_\beta_1\) preferentially contribute to the adhesion process whereas engagement of GPVI triggers signaling events leading to platelet activation [67]. GPVI is non-covalently associated with the Fe receptor \(\gamma\)-chain and signals through the platelet via stimulation of multiple non-receptor tyrosine kinases. Interestingly, part of this signaling pathway may be common to GPIb\(\alpha\) activation, and signals coming from these two receptors contribute to the activation of the receptor function of GP Ib-IIIa and platelet aggregation. Modulation of the GPVI receptor function is becoming an attractive target as platelets from GPVI-deficient animals, human platelets expressing low levels of GPVI, or platelets treated with a GPVI antibody, while unable to support thrombus growth [67–69], are nonetheless able to adhere on the collagen surface (via \(\alpha_\beta_1\) integrin and potentially another collagen receptor [70]) minimizing the effects on hemostasis [71,72]. GPIb\(\alpha\) is a high shear rate-dependent thrombosis receptor that affects recruitment of platelets at sites of vascular injury (on the collagen present in the subendothelium and on adhering platelets) with minor impact on venous thrombotic process. Modulation of the VWF/GPIb\(\alpha\) axis has been the subject of many investigations with promising animal experimental results, but severe thrombocytopenia has been associated with the use of antibodies against GPIb\(\alpha\), thus reducing the general interest of the scientific community for several years. Nevertheless, novel strategies targeting the VWF/GPIb\(\alpha\) axis through snake venom proteins cleaving GPIb\(\alpha\), VWF peptides or antibodies against VWF are reviving this strategy.

Signaling pathways

The extensive repertoire of platelet functions, while initiated by receptors, is regulated by signal transduction pathways. While these pathways have not been known as drug discovery targets, two observations suggest that they are worthy of consideration. First, the remarkable success of Gleevec, an Abelson tyrosine kinase inhibitor, has proven efficacy in the treatment of chronic myelogenous leukemia and other cancers. While the multiple functions of this kinase in diverse cell types predicted toxicity, clinical data have shown that the benefits far outweigh the liabilities. The success of this drug suggests that signal transduction pathways, though redundant for multiple signaling systems in diverse cell types, are worthy of consideration as therapeutic drug discovery targets. This conclusion is supported by the analysis of numerous gene-targeted mouse strains which have led to the surprising conclusion that the phenotype achieved by the disruption of any specific gene is often limited, even for genes involved in signal transduction. A second observation is that the platelet stimuli often induce diverse responses. For example, any one of the primary platelet agonists are capable of producing a spectrum of responses which could have pathological implications, e.g. aggregation caused by the activation of the receptor function of GP Ib-IIIa, expression of procoagulant activity of prothrombinase or FXase to catalyze the production of thrombin, generation of vasoactive substances such as TXA2 and serotonin to induce vasoconstriction, release of proinflammatory proteins like sCD40L, RANTES and TGF-\(\beta\) to affect vascular inflammation including the progression of atherosclerosis, the release of growth factors such as PDGF to affect vascular remodeling, and the activation of secondary aggregation receptors such as SLAM, CD84 and the ephrins to stabilize thrombi and cause vascular occlusions. As many of these responses would be expected to be regulated by a specific pathway, it is reasonable to expect that these responses could be individually regulated. If true, this approach could inhibit platelet-dependent pathologies without compromising primary hemostasis. One potential example is PI-3 kinase and the regulation of the adhesive function of GP Ib-IIIa [73].

The roles of secondary signaling events downstream of platelet surface receptors have been elucidated through gene-targeting studies in mice, and subsequent evaluation of their platelet phenotypes using both \textit{in vitro} and \textit{in vivo} techniques. As certain key platelet agonists such as ADP, thrombin, and TXA2 all activate platelets through G protein-coupled receptors, genetic targeting of individual \(\alpha\)-subunits of G proteins has been a successful strategy in studying platelet signaling downstream of receptor activation. Characterization of platelets from mice lacking \(G_{\alpha}\), \(G_{\beta}\), and \(G_{\gamma}\), identified these three proteins as key mediators for ADP and TXA2 receptor [27,74–76]. Targeting of additional subunits (\(G_{\alpha}\), \(G_{\beta2}\), and \(G_{\gamma2}\)) showed little or no effect on platelet phenotype, which could be due to lack of coupling of these subunits to critical platelet receptors, or due to redundancy in the signaling pathways. Thrombin signaling has been shown to be affected by lack of \(G_{\alpha}\), \(G_{\gamma}\), and
G_{13}, to varying extents. In mice lacking both G_q and G_{13}, no platelet activation was possible by ADP, TXA_2, or thrombin [27], suggesting that at least G_q or G_{13} is required to induce some activation, and that activation of G_q-type proteins alone is not sufficient for activation of mouse platelets. In these G_q/G_{13} double-deficient platelets, adhesion of platelets to collagen was not affected; however, aggregation in response to collagen fibrils as well as formation of stable aggregates on collagen-coated surfaces was completely eliminated. In addition to targeting of G protein subunits, targeting molecules involved in kinase signaling pathways have resulted in mice with impaired platelet functions, which is not unexpected given the fact that kinases of the src family (Csk, src) have been shown to be physically associated with the cytoplasmic domain of GP IIb-IIIa [77]. The platelet phenotype of mice lacking the tyrosine kinase syk, critical for downstream signaling through the collagen receptor GPVI, and also activated during outside-in signaling and activation of GP IIb-IIIa, exhibited defects in platelet activation induced by ADP ± epinephrine [78]. Mice lacking the adapter protein SLP-76, which is on the syk signaling pathway, have been shown to have defects in GP IIb-IIIa signaling and collagen receptor responses [79]. The important role of downstream signaling molecules identified through gene-targeting studies may provide new opportunities for therapeutic intervention for blockade of platelet activation, thrombus formation, and adhesion.

**Evolving paradigm on the relationship of thrombosis to inflammation**

Not only are platelets critical players in mediating thrombosis, but recently their role in inflammation has become more appreciated. Although it is widely accepted that inflammatory activities that orchestrate the progression of atherosclerosis are derived from ‘traditional’ inflammatory cells such as monocytes and neutrophils, emerging data suggest that products released from platelets during thrombosis are actively involved in this process and that platelets are a primary source of inflammatory proteins within the circulation. For example, Schober et al. [80] reported that platelets deposit RANTES onto endothelial cells in the injured vessel wall, and that this interaction is mediated by P-selectin, a surface receptor mediating the attachment of platelets to leukocytes and endothelium. Additional work from this laboratory showed that infusion of activated platelets into the apoE^{-/} mouse greatly enhanced the rate of atherosclerotic lesion progression [81]. The exposure of P-selectin following platelet activation is a key mediator of platelet–leukocyte interaction, and facilitates atherosclerotic lesion development, as demonstrated by Burger and Wagner [82]. In patients with acute MI, platelet–leukocyte interaction is increased compared with controls, and P-selectin levels have been shown to remain increased for at least a month following initial presentation in ACS patients with non-ST segment elevation [83], and even in patients with stable coronary artery disease. Antiplatelet therapy with a P2Y_{12} antagonist plus aspirin was shown to decrease platelet–monocyte interactions that occur after coronary stenting [84], an effect not observed with anticoagulants plus aspirin, suggesting that P2Y_{12} antagonism had an anti-inflammatory effect distinct from its antithrombotic mode of action. Targeting of CD40L, another platelet-derived inflammatory protein, either through a blocking antibody [85] or via gene-targeting [86], greatly inhibited lesion progression in either LDLR^{-/} or apoE^{-/} mice, respectively. Soluble CD40L (sCD40L), the shed hydrolytic product of CD40L, 95% of which is platelet-derived, has been shown to be a primary risk factor for atherosclerosis/ thrombosis [87]. In addition, the binding of platelet-derived sCD40L to endothelial cells can lead to the expression of tissue factor, a potent procoagulant. Clopidogrel has been shown to inhibit ADP-induced CD40L expression, and to lower CD40L levels in patients undergoing PCI [88], while aspirin does not. Thus, some forms of antiplatelet therapy, including P2Y_{12} inhibition, can inhibit platelet pro-inflammatory responses. Finally, recent data show that targeting of GP Ib-IX-V complex, a platelet adhesion receptor, in the apoE^{-/} mouse blocks leukocyte recruitment and the development of atherosclerotic lesions [89]. Thus, studies of the role of platelets in inflammation may provide new potential targets for CVD through inhibition of atherosclerosis and/or thrombosis.

**Platelet monitoring**

The pharmaceutical industry and drug approval agencies expect that the early inroads into personalized medicine in the administration of selected chemotherapies will ultimately extend to all drug classes. These early examples include screening for HER2 positive individuals in the treatment of breast cancer with Herceptin and the screening for EGF receptor for the treatment of lung cancer with gefitinib [90]. More generally, by the time this manuscript is published, the CYP450 screen will most likely be available to detect the various isoforms of P450 which will be useful in projecting drug levels in individuals treated with a wide variety of drugs. While such strategies will be of value in selecting and dosing antithrombotics, particularly for chronic use, access to blood of patients treated with antithrombotics continues to provide the best opportunity for monitoring the effect of any therapy or any combination of therapies on each patient being treated. While suitable assays are available to monitor anticoagulants, the monitoring of antiplatelet drugs is not routinely performed.

Evaluations of data from the current antithrombotics and their limitations have defined the assays required to bring personalized medicine to the patient treated with antiplatelet drugs to prevent arterial thrombosis. First, platelet function should be monitored in the context of thrombosis. Platelet thrombosis in vivo is initiated by adhesive proteins exposed on the vessel wall and stable thrombi result following adhesion, activation, aggregation, and thrombus stabilization, all occurring under conditions of shear. As thrombus stability is one of the issues, continuous monitoring of thrombus formation is essential for determining the effects of drugs that effect targets
involved in thrombus stability, for example, in prostanoid metabolism and in ADP release. Although methods currently available such as light transmittance aggregometry, the Ultegra Rapid Platelet Function Assay and platelet activation markers such as P-selectin expression are effective in monitoring the ability of end products of any one of these pathways to activate platelets, they are ineffective in monitoring thrombosis, the physiological response of platelets to thrombogenic surfaces under shear. Furthermore, while the PFA-100 device is capable of monitoring the time required for a platelet plug to form in apertures coated with ADP or collagen, it does not provide a continuous monitoring of the thrombotic process. A second requirement for the personalized monitoring of drugs to prevent arterial thrombosis is that it should not only be responsive to diverse drug classes but also be capable of determining the net effect on thrombosis achieved by combinations of antithrombotic therapies. At the present time, the four drug classes used to treat patients at risk for arterial thrombosis, aspirin, GP IIb-IIIa antagonists, clopidogrel and anticoagulants, are used in combinations not properly evaluated for their net effect on thrombosis. Drugs against additional platelet and coagulation protein targets will become available. Required are technologies that will readily permit evaluation of how combinations of these drugs affect thrombosis in patients receiving these drugs. A third requirement is that the method must be capable of monitoring individual differences in response to therapy. As outlined above, individual differences in response to aspirin and clopidogrel have been observed – differences which appear to affect clinical outcome. While currently available techniques such as light transmittance aggregometry, Ultegra, or PFA-100 are useful in monitoring responsiveness to both of these drugs as monotherapy, they are ill-suited to measure individual differences when combinations of drugs are employed, the emerging norm. It is also anticipated that this problem would be amplified when drugs against additional targets would be introduced. Finally, as the anticoagulants routinely used in blood collection such as citrate or direct thrombin inhibitors such as PPACK affect thrombosis, the monitoring method must be capable of assaying non-anticoagulated samples of blood. Perhaps the best example of anticoagulant interference in antithrombotic monitoring is in the development of GP IIb-IIIa antagonists where citrate anticoagulation markedly overestimates antiaggregatory activity [91]. The optimal method for monitoring individual thrombotic potential must be capable of either performing the assay in the absence of anticoagulants or of being able to determine how any given anticoagulant affects the assay.

Use of perfusion chamber technology has perhaps provided the best hope of measurement of the thrombotic potential of individuals being treated with combinations of antithrombotic drugs. Perfusion chambers were designed 30 years ago in order to characterize the thrombotic process under shear conditions. The different types of perfusion chambers described in the literature can be classified according to their geometry (circular, annular, flat chambers) or the surfaces (blood vessels, isolated proteins) exposed to flowing blood. These techniques confer the advantage of studying platelet interactions with a thrombogenic surface under specific conditions of shear rate with either non-anticoagulated or anticoagulated blood. Major contributions to the field of thrombosis have originated from use of perfusion chambers. For example, the critical role of VWF and its interactions with GP Ib and GP IIb-IIIa to mediate platelet adhesion and thrombus growth under arterial shear rates, the involvement of GP VI and of the integrin α2β1 in mediating platelet adhesion and activation on collagen. Inhibitors of P2Y12 and Cox-I have also demonstrated antithrombotic activities in this system [92]. However, perfusion chambers are mostly utilized by academic institutions or by pharmaceutical and biotechnology companies in order to identify or validate targets and to develop antithrombotic drugs. Several limiting factors have prevented their use as a bedside device for monitoring drug efficacy in clinical trials – the skill required to determine thrombus size was not readily available in clinical settings; quick readouts for the patient were not available; end point quantifications left investigators without knowledge of the kinetics of thrombus formation, the more critical information.

Results from several laboratories, however, have made progress in modifying these devices to circumvent these difficulties. Figures 3 and 4 illustrate the utility of monitoring the kinetics of thrombosis in perfusion chamber assays. In one instance, using non-anticoagulated samples of blood, we have shown that inhibition of P2Y12, Cox-1, or FXa did not significantly reduce thrombus size after a 4-min perfusion period over a collagen-coated surface. However, when more than one of these targets was inhibited, pronounced anti-thrombotic activity was observed. In another experiment, when human blood was anticoagulated with an FXa inhibitor and perfused through a chamber in the real-time assay, we observed that P2Y12 antagonism with clopidogrel did not affect the

![Fig. 3. Synergy between P2Y12 antagonism, Factor Xa inhibition and aspirin. As indicated unanticoagulated blood was treated with an inhibitor for P2Y12 (100 μM 2MeSAMP) or Factor Xa (10 μM C921–78). Aspirin-treated was from aspirin-treated individuals. The treated blood was perfused through a chamber coated with type III collagen at 1000 s⁻¹ for 4 min and quantified as described [23].](https://example.com/fig3.png)
initial thrombus growth triggered by fibrillar type III collagen under arterial shear rates. However, clopidogrel caused the thrombi formed during the first 3 min of perfusion to dissociate. Control thrombi formed in the absence of clopidogrel were stable and continued to grow. This demonstrates the limitations of end point analysis as the measured antithrombotic activity is dependent on the time of analysis. Retrospectively, this explains an apparent discrepancy found in the evaluation of the phenotype of P2Y<sub>12</sub>−/− mice [57]. P2Y<sub>12</sub>−/− mice demonstrate a cyclic thrombotic process in vivo, but only a qualitative difference (i.e. more loosely packed thrombi) was observed after perfusion of non-anticoagulated blood for 2.5 min over type III collagen. Evaluation of mono- and combination therapies in this assay confirmed the antithrombotic efficacy of the different anti-platelet therapies, with GP IIb-IIIa antagonists being inhibitors of thrombus growth, aspirin and P2Y<sub>12</sub> antagonist destabilization agents, the combination aspirin + P2Y<sub>12</sub> antagonism showing a faster destabilization activity (Fig. 4). Thus, perfusion chamber technology is suited to meet the requirements of personalized medicine for individuals receiving antithrombotic therapies as the measurement is on thrombosis, it is responsive to diverse drug classes and it can be performed in the absence of anticoagulants. Future discoveries are required to adapt such technologies to devices that are readily available to individual patients. Finally, several laboratories have reported measurements of the inflammatory activities of platelets, e.g. sCD40L plasma levels, P-selectin expression, formation of platelet–leukocyte complexes. As recent data show that the inflammatory activity of platelets is important in the progression of atherosclerosis, it would also be desirable to develop methods to rapidly quantitate the platelet inflammatory activity in patients.

Application of this personalized medicine approach to antithrombotic therapies does have significant hurdles to overcome before it can be used to reliably modify therapy. First, a bedside monitor of the thrombotic potential of individual patients needs to be developed. Second, recognizing that individuals will undoubtedly be heterogeneous with respect to vessel wall thrombogenicity, including the local shear environment, results using this device need to be correlated with clinical outcomes.

Conclusions

The current repertoire of drugs for the treatment of patients at risk for arterial thrombosis (e.g. ACS, diabetes, poststroke, peripheral artery disease, post-AMI) currently includes four classes of drugs – aspirin, GP IIb-IIIa antagonists, thienopyridines, and anticoagulants. Although each of these drug classes has proven efficacies for different indications, each has limitations that continue to permit thrombotic events during their use. In addition, emerging data suggest that a significant percentage of individuals treated with aspirin or clopidogrel do not receive the expected therapeutic benefit from therapy because of a decreased responsiveness by their platelets. Future directions in addressing these limitations will proceed in two parallel directions. On the one hand, it can be anticipated that new drugs, either offering improvements against known, validated targets, or against recently identified targets, will be forthcoming. Recognizing that platelets are now known to be directly involved in vascular inflammation including that which leads to the progression of atherosclerotic disease, it can be anticipated that some of these new therapeutic strategies will not only better address arterial thrombosis, but also inhibit the ability of platelets to deliver inflammatory proteins and growth factors which affect atherosclerotic lesion development. On the other hand, it has now become apparent that improvements are required in the devices used to monitor the thrombotic potential of individuals receiving therapy, both for the development of new antithrombotic drugs and to measure the effectiveness of combined antithrombotic therapies. It would appear that the most effective device is that which measures thrombosis in real time, is accessible to the patient at the point of drug administration, and can be performed in the absence of anticoagulation. Such a device would be capable of monitoring the activities of new classes of antithrombotics, of measuring variances of individual responses, and in evaluating the effectiveness of combined antithrombotic therapies.

References


Treat ing arterial thrombosis


