

## Inherited defects in platelet signaling mechanisms

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Patients with an inherited bleeding disorder and abnormalities in the routinely performed platelet aggregation studies are not uncommonly encountered in clinical practice. However, in the vast majority of these patients, the underlying molecular mechanisms leading to the platelet dysfunction are unknown. The best recognized entities such as thrombasthenia, Bernard–Soulier Syndrome, and the dense granule storage pool deficiency have provided unparalleled insights in platelet physiology, but are distinctly uncommon. Evidence has become available that specific abnormalities in platelet signaling mechanisms may be the basis of platelet dysfunction in some patients. They constitute the focus of this review.

Selected aspects of normal platelet mechanisms in hemostasis are shown in Fig. 1. When the blood vessel is injured, platelets adhere to exposed subendothelium by a process which involves the interaction of a plasma protein, von Willebrand factor (VWF), and a specific protein complex on the platelet surface, glycoprotein Ib-IX-V complex (Fig. 1). Adhesion is followed by recruitment of additional platelets which form clumps (aggregation); this involves binding of fibrinogen to specific platelet surface receptors – a complex comprising glycoproteins IIb–IIIa (GPIIb–IIIa), and a process that is signal transduction dependent. Activated platelets release contents of their granules (secretion or release reaction), such as adenosine diphosphate (ADP) and serotonin from the dense granules, which cause recruitment of additional platelets. In addition, platelets play a major role in coagulation mechanisms; several key enzymatic reactions occur on the platelet membrane lipoprotein surface. Several agonists interact with specific receptors on platelet surface to induce responses including a change in platelet shape from discoid to spherical (shape change), aggregation, secretion, and thromboxane A<sub>2</sub> (TxA<sub>2</sub>) production. Other agents, such as prostacyclin, inhibit these responses. Ligation of the platelet receptors initiates the production or release of several intracellular messenger molecules including Ca<sup>2+</sup> ions, products of phosphoinositide (PI) hydrolysis by phospholipase C (diacylglycerol, DG, and inositol 1,4,5-triphosphate, InsP<sub>3</sub>), TxA<sub>2</sub> and cyclic nucleotides (cAMP). These induce or modulate the various platelet responses of Ca<sup>2+</sup>

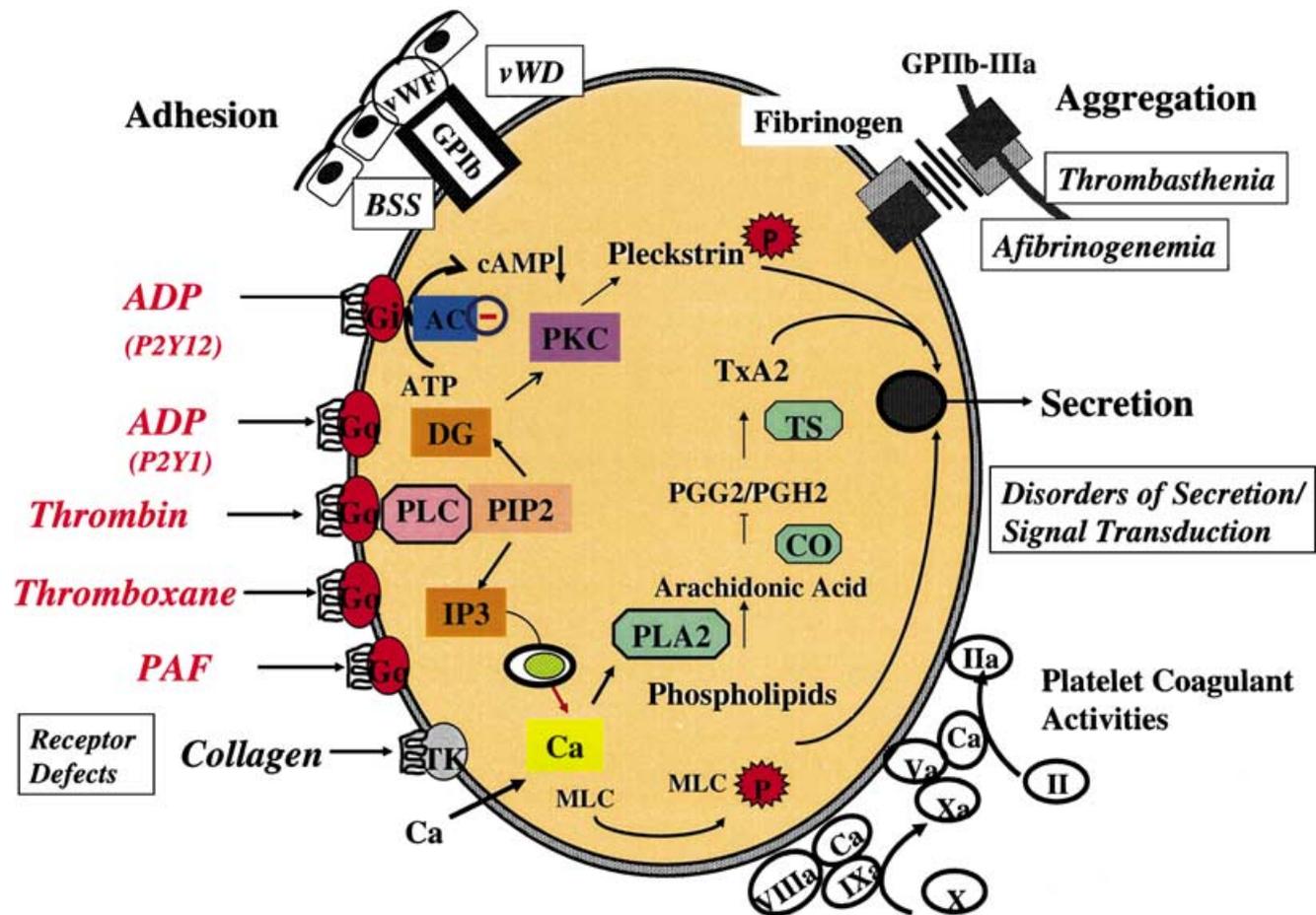
mobilization, protein phosphorylation, aggregation, secretion and liberation of arachidonic acid. The interaction between the agonist receptors and the key intracellular effector enzymes (e.g. phospholipase A<sub>2</sub> (PLA<sub>2</sub>) and phospholipase C (PLC), adenylyl cyclase) is mediated by a group of GTP-binding proteins which are modulated by GTP. As in most secretory cells, platelet activation results in a rise in cytoplasmic ionized calcium concentration; InsP<sub>3</sub> functions as a messenger to mobilize Ca<sup>2+</sup> from intracellular stores. Diacylglycerol activates protein kinase C (PKC) and this results in the phosphorylation of a 47-kDa protein pleckstrin. PKC activation is considered to play a major role in platelet secretion and in the activation of GPIIb–IIIa. Numerous other mechanisms, such as phosphorylation of proteins by non-receptor tyrosine kinases, also play a role in signal transduction. A detailed description of the platelet activation mechanisms is beyond the scope of this review.

### Congenital disorders of platelet function

In general, congenital disorders of platelet function are characterized by highly variable mucocutaneous bleeding manifestations and excessive hemorrhage following surgical procedures or trauma. A majority of patients, but not all, have a prolonged bleeding time. Platelet aggregation and secretion studies provide evidence for the defect but are not generally predictive of the severity of clinical manifestations. The platelet dysfunction in these patients arises by diverse mechanisms [1]. Table 1 provides a classification based on the platelet functions or responses that are abnormal (Fig. 1). In patients with defects in platelet–vessel wall interactions, adhesion of platelets to subendothelium is abnormal. The two disorders in this group are the von Willebrand disease (VWD), due to a deficiency or abnormality in plasma VWF, and the Bernard–Soulier syndrome, where platelets are deficient in GPIb (and GPV and IX) and platelet–vWF binding is abnormal. Disorders characterized by abnormal platelet–platelet interactions (aggregation) arise because of an absence of plasma fibrinogen (congenital afibrinogenemia) or because of a quantitative or qualitative abnormality of the GPIIb–IIIa complex (Glanzmann thrombasthenia). Patients with defects in platelet secretion and signal transduction are a heterogeneous group lumped together for convenience of classification rather than on the basis of an understanding of the specific underlying abnormality. The major common characteristic in these patients, as currently perceived, is an inability to release intracellular granule (dense) contents upon activation of platelet-rich plasma (PRP) with

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**Fig. 1.** A schematic representation of selected platelet responses to activation and the congenital disorders of platelet function. Abbreviations: AC, adenylyl cyclase; BSS, Bernard–Soulier syndrome; CO, cyclooxygenase; DAG, diacylglycerol; G, GTP-binding protein; IP3, inositoltrisphosphate; MLC, myosin light chain; MLCK, myosin light chain kinase; PIP2, phosphatidylinositol biphosphate; PKC, protein kinase C; PLC, phospholipase C; PLA2, phospholipase A2; TK, tyrosine kinase; TS, thromboxane synthase; VWF, von Willebrand factor; VWD, von Willebrand disease. The Roman numerals in the circles represent coagulation factors. (Modified with permission from Rao A.K. *Am J Med Sci* 1998; **316**: 69–77.)

agonists such as ADP, epinephrine, thromboxane A2 and collagen. In aggregation studies the second wave of aggregation is blunted or absent. A small proportion of these patients have a deficiency of dense granule stores (storage pool deficiency). In some of the other patients, the impaired secretion results from aberrations in the signal transduction events that govern end responses such as secretion and aggregation; this review will focus on these patients. Lastly, are the patients who have an abnormality in interactions of platelets with proteins of the coagulation system; the best described is the Scott syndrome [2]. In addition to the above groups, there are patients who have abnormal platelet function associated with systemic disorders, such as Down's syndrome, where the specific aberrant platelet mechanisms are unclear.

#### Disorders of platelet secretion and signal transduction

As an unifying theme, patients lumped in this remarkably heterogeneous group generally manifest decreased aggregation and absence of the second wave of aggregation upon stimula-

tion of PRP with ADP and epinephrine and impaired secretion of granule contents; responses to collagen, thromboxane analog U46619, arachidonic acid, and platelet-activating factor (PAF) may also be impaired. Platelet function is abnormal in these patients either when the granule contents are diminished (storage pool deficiency, SPD) or when there are aberrations in the activation mechanisms governing aggregation and secretion, the easily discernible but relatively late responses following activation (Table 1).

#### Deficiency of granule stores: storage pool deficiency

Patients with storage pool deficiency (SPD) have deficiencies in platelet content of dense granules ( $\delta$ -SPD), alpha-granules ( $\alpha$ -SPD) or both types of granules ( $\alpha\delta$ -SPD) [1,3]. The Quebec platelet disorder is an autosomal dominant disorder associated with abnormal proteolysis of  $\alpha$ -granule proteins, deficiency of platelet  $\alpha$ -granule multimerin (a factor V binding protein), and markedly impaired aggregation with epinephrine as a striking feature [3].

**Table 1** Classification of congenital disorders of platelet function

1. Defects in platelet–vessel wall interaction (disorders of adhesion)
  - a. von Willebrand disease (deficiency or defect in plasma VWF)
  - b. Bernard–Soulier syndrome (deficiency or defect in GPIb)
2. Defects in platelet–platelet interaction (disorders of aggregation)
  - a. Congenital afibrinogenemia (deficiency of plasma fibrinogen)
  - b. Glanzmann’s thrombasthenia (deficiency or defect in GPIIb–IIIa)
3. Disorders of platelet secretion and signal transduction mechanisms
  - a. Abnormalities of granules
    - i. Storage pool deficiency
    - ii. Quebec platelet disorder
  - b. Signal transduction defects (primary secretion defects)
    - i. Defects in platelet-agonist interaction (receptor defects)  
Receptor defects: thromboxane A<sub>2</sub>, collagen, ADP, epinephrine
    - ii. Defects in G-protein activation  
G $\alpha$ q deficiency
    - iii. Defects in phosphatidylinositol metabolism  
Phospholipase C- $\beta$ 2 deficiency
    - iv. Defects in calcium mobilization
    - v. Defects in protein phosphorylation (pleckstrin)
  - c. Abnormalities in arachidonic acid pathways and thromboxane A<sub>2</sub> synthesis
    - i. Impaired liberation of arachidonic acid
    - ii. Cyclooxygenase deficiency
    - iii. Thromboxane synthase deficiency
  - d. Defects in cytoskeletal regulation  
Wiskott–Aldrich syndrome
4. Disorders of platelet coagulant–protein interaction  
Scott syndrome

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### Defects in platelet signal transduction (primary secretion defects)

Signal transduction mechanisms encompass processes that are initiated by the interaction of agonists with specific platelet receptors and include responses such as G-protein activation and activation of effectors such as PLC and PLA<sub>2</sub>. Simplistically, if the key components in signal transduction are the surface receptors, the G-proteins, and the effectors, evidence now exists for specific human platelet abnormalities at each of these levels. It is only a matter of time before deficiencies are documented in the various specific platelet signaling proteins/pathways.

### Defects in platelet–agonist interaction: receptor defects

These patients are characterized by impaired platelet responses resulting from an abnormality of platelet surface receptors for a specific agonist. Such receptor defects have been documented for TxA<sub>2</sub>, collagen, ADP, and epinephrine. One patient has been described with diminished responses to PAF alone [4]. Because ADP and TxA<sub>2</sub> play a synergistic role in the platelet responses to several agonists, patients with defects in the ADP or TxA<sub>2</sub> receptor have impaired responses to other agonists, including collagen and thrombin.

### Thromboxane A<sub>2</sub> receptor defect

Several patients have been previously described where the platelet dysfunction has been attributed to an abnormality in

the platelet TxA<sub>2</sub> receptor (see reference [1] for review) but the molecular defects have not been delineated. Specific mutations in the platelet TxA<sub>2</sub> receptor have been documented by Hirata *et al.* [5] who described an Arg60 to Leu mutation in the first cytoplasmic loop of the TxA<sub>2</sub> receptor in two unrelated patients with a mild bleeding disorder of autosomal dominant inheritance. This Arg60 corresponds to a highly conserved basic residue among G-protein-coupled receptors [5]. Aggregation responses to several agonists were impaired with the exception of thrombin [6]. The binding of TxA<sub>2</sub> analogs to platelets was normal [6,7]. GTPase activity on activation with a TxA<sub>2</sub> analog, but not thrombin, was diminished [7,8] suggesting a defect in TxA<sub>2</sub> receptor-G-protein coupling. TxA<sub>2</sub>-induced activation of PLC (measured as Ca<sup>2+</sup> mobilization, and InsP<sub>3</sub> and phosphatidic acid formation) was impaired while PLA<sub>2</sub> activation and TxA<sub>2</sub> production were normal. Less than half the normal number of TxA<sub>2</sub> receptors are sufficient for irreversible aggregation with TxA<sub>2</sub> agonist [9]. Therefore, the finding that the aggregation responses were impaired in the heterozygous family members [5] suggests a dominant negative effect of the mutation. Of note, absent aggregation response to TxA<sub>2</sub> have been observed in patients who do not have evidence for a defect of TxA<sub>2</sub> receptor [10]. Lastly, in line with observations in the patients, TxA<sub>2</sub> receptor knockout mice have a mild bleeding disorder and impaired platelet aggregation responses to TxA<sub>2</sub> analogs as well as collagen [11].

### Collagen receptor defects

Patients have been reported with mucocutaneous bleeding manifestations and selective impairment in platelet–collagen interaction. In the current models of platelet–collagen interactions, collagen binds initially to either the GPIa–GPIIa ( $\alpha$ 2 $\beta$ 1) or GPVI, leading to subsequent binding to the other receptor which serves to reinforce adhesion and generation of intracellular signals including Ca<sup>2+</sup> mobilization and protein phosphorylation [12]. Platelets from the patient described by Nieuwenhuis *et al.* [13,14] had ~15–25% of normal platelet GPIa and failed to aggregate with collagen, or adhere and spread normally to subendothelial surfaces. In another patient described by Kehrel *et al.* [15], collagen-induced platelet aggregation was markedly reduced, and the platelets were deficient in GPIa and thrombospondin. In both patients, the bleeding times were prolonged and platelet aggregation responses to other agonists were preserved. Selective impairment in collagen responses and a mild bleeding disorder have also been related to a deficiency of platelet GPVI [16–18]. GPVI-deficient platelets have been reported to have impaired collagen-stimulated activation of Syk but not c-Src [19].

GPIV (CD36) has also been implicated in platelet–collagen interactions but not fully established. Platelets lacking GPIV have been reported to have reduced adhesion to collagen in flowing whole blood [20]. However, individuals lacking platelet GPIV in the Japanese population (approximately 3% of the population) and the US population (approximately 0.3%) do not have a bleeding disorder [21] and collagen-induced platelet

aggregation [21],  $\text{Ca}^{2+}$  mobilization, and tyrosine phosphorylation [22] have been normal. In other studies [23], GPIV-deficient platelets aggregated normally in response to collagens type I and III but not to type V collagen. Moreover, adhesion and subsequent aggregate formation of such platelets on types I, III and IV collagens were normal under static or flow conditions whereas adhesion to type V collagen was reduced under static conditions [24]. In several GPIV-deficient subjects a Pro90 to Ser mutation has been reported in the GPIV gene [25] and GPIV mRNA has been detected in platelets [25,26]. In studies by Kehrel *et al.* [23] platelets-deficient in GPVI but not GPIV failed to aggregate in response to collagen-related peptides.

Lastly, in contrast to other agonists (e.g. ADP, thrombin, TxA<sub>2</sub>) which induce their platelet responses via G-protein-coupled receptors, collagen-induced responses are considered mediated by activation of tyrosine kinase Syk and PLC- $\gamma$ 2 [12]. However, platelets deficient in G $\alpha$ q [27] PLC- $\beta$ 2 [28], ADP receptor [29] or TxA<sub>2</sub> receptor [6] have impaired collagen-induced responses, suggesting that collagen also activates G-protein-mediated mechanisms. This may be secondary to the effects of the released ADP or TxA<sub>2</sub>.

### Defect in platelet ADP receptors

ADP interaction with platelets is mediated by multiple receptors (P2Y<sub>1</sub>, P2Y<sub>12</sub> (formerly P2TAC), P2X<sub>1</sub>) which elicit distinct responses [30]. P2Y<sub>1</sub> receptors are responsible for PLC activation, intracellular  $\text{Ca}^{2+}$  mobilization and shape change while P2Y<sub>12</sub> receptors mediate inhibition of cAMP formation by adenylyl cyclase. ADP-induced aggregation requires coactivation of both P2Y<sub>1</sub> and P2Y<sub>12</sub> receptors. P2X<sub>1</sub> receptors function as cation channels. Cattaneo *et al.* [29,31] and Nurden *et al.* [32] have described patients who have blunted (but not absent) platelet aggregation response to ADP and diminished ability of ADP to suppress PGE<sub>1</sub>-induced elevation in cAMP; ADP stimulated shape change was normal. ADP stimulated  $\text{Ca}^{2+}$  mobilization [29] and tyrosine phosphorylation in response to ADP and TxA<sub>2</sub> [33] have been reported to be abnormal in the patient studied. Aggregation in response to a TxA<sub>2</sub> analog and lower concentrations of collagen were also impaired. The platelet binding of radiolabeled ADP [29] or the ADP analog 2-methylthio-ADP [31,32] was decreased in these patients. Electron microscopic studies show [34] that the platelet aggregates formed in these patients following stimulation with high dose ADP were composed of loosely bound platelets with few contact points. A decreased platelet 2-methylthio-ADP binding has also been reported [35] in additional patients with impaired aggregation and secretion in response to several agonists including ADP.

Recent studies have delineated the genetic defect in several of these patients. Studies in one of these patients [32] provide evidence of one mutant allele in the P2Y<sub>12</sub> gene with deletion of two nucleotides in the coding region (at amino acid 240) resulting in a frame shift and a premature stop codon [36]. Interestingly, although this patient has one P2Y<sub>12</sub> allele with normal coding region, the patient's platelets have an almost

complete lack of P2Y<sub>12</sub> ADP receptors. The P2Y<sub>12</sub> transcripts in the platelet RNA are derived entirely from the mutant allele suggesting a repression of the wild-type allele. In contrast, platelets from the patient's daughter have an intermediate number of ADP-binding sites [32], normal ADP responses, and one frame-shifted allele and one wild-type allele, suggesting that the mutant allele does not act in a dominant negative manner. Studies in a second patient [37] have revealed a compound heterozygous state with one allele containing a G to A transition resulting in an Arg256 to Gln codon substitution, and the other allele containing a C to T transition and Arg265 to Trp codon substitution. Both mutations occurred in the third extracellular loop of the receptor. Expression studies in CHO cells revealed that neither mutation affected the translocation of the P2Y<sub>12</sub> receptor to cell surface but ADP-induced inhibition of adenylyl cyclase was partially inhibited, indicating presence of a dysfunctional receptor on the cell surface. In three other patients [29,31], homozygous deletions of one to two base pairs have been demonstrated [38] in the P2Y<sub>12</sub> locus, resulting in premature termination and a lack of demonstrable protein in the platelet lysates. In line with the studies in patients, P2Y<sub>12</sub> null mice have shown a prolonged bleeding time, and platelets that aggregate poorly in response to ADP, retain normal shape change but fail to inhibit adenylyl cyclase [39].

In addition to the patients mentioned above with the P2Y<sub>12</sub> ADP receptor deficiency, one patient has been briefly described [40] with a defect in the P2Y<sub>1</sub> platelet receptor which is coupled to PLC- and ADP-induced calcium mobilization. This patient also had impaired platelet aggregation in response to ADP and other agonists. P2Y<sub>1</sub>-deficient mice have decreased platelet aggregation, a prolonged bleeding time and resistance to thromboembolism [41,42]. Lastly, Oury *et al.* [43] have reported a patient with a selective impairment of ADP-induced aggregation associated with a dominant negative mutation in P2X<sub>1</sub> receptor due to deletion of one leucine residue within a stretch of four leucine residues in the second transmembrane domain. The association of a bleeding disorder with alterations in this receptor (an ATP-gated ion channel) suggests its hitherto unrecognized physiological role in hemostasis.

### Selective impairment in platelet responsiveness to epinephrine

Aggregation responses to epinephrine, mediated by  $\alpha$ 2-adrenergic receptors ( $\alpha$ 2AR), may be variable or even impaired in some presumably normal individuals. In one study [44], the second wave of aggregation was noted to be absent in 10–15% of normal subjects. Studies in twins suggest that platelet  $\alpha$ 2AR are under genetic control [45]. Scrutton *et al.* [46] reported depressed platelet aggregation response to epinephrine in five apparently normal volunteers and indicated that the defect was familial. However, in four of the index cases, platelet responses to other agonists such as collagen, vasopressin, and ADP were also impaired. Rao *et al.* [47] have described a family in which several members had impaired aggregation and secretion in response to only epinephrine associated with decreased number

of platelet  $\alpha_2$ AR. Three of the family members had a history of easy bruising with minimally prolonged bleeding times. Although aggregation response was impaired, epinephrine inhibition of adenylate cyclase was normal indicating that the receptor requirements for these two platelet responses are different. Although other families with an epinephrine defect have been reported [48] the relationship of the selective epinephrine defect to bleeding manifestations still needs to be defined. It is tantalizing to speculate that in some of these patients with bleeding symptoms, the isolated impaired aggregation response to epinephrine may be related to the Quebec Platelet Syndrome [3]. Interestingly, mice deficient in  $G_{\alpha z}$ , a member of the Gi family of G-proteins, have impaired platelet aggregation and ability to inhibit cAMP formation in response to epinephrine, but do not bleed and are resistant to fatal thromboembolism [49].

### Defects in GTP-binding proteins

GTP-binding proteins are a group of heterotrimeric (consisting of  $\alpha$ ,  $\beta$  and  $\gamma$  subunits) proteins that constitute the link between surface receptors and intracellular enzymes. Because of their modulating role as molecular switches, G-proteins constitute an important potential locus for aberrations leading to platelet dysfunction. Convincing evidence for the existence of such a defect has been provided by Gabbeta *et al.* [50] in a patient with a mild bleeding disorder, abnormal aggregation and secretion in response to a number of agonists, and diminished GTPase activity (a reflection of  $\alpha$ -subunit function) on activation. The binding of  $^{35}\text{S}$ -GTP $\gamma$ S to platelet membranes was diminished, and there was a selective decrease in platelet membrane  $G_{\alpha q}$  with normal levels of  $G_{\alpha i2}$ ,  $G_{\alpha 12}$ ,  $G_{\alpha 13}$  and  $G_{\alpha z}$ . This patient has abnormalities in other downstream signaling events: activation of the GPIIb-IIIa complex,  $\text{Ca}^{2+}$  mobilization [51], and release of arachidonic acid from phospholipids on platelet activation [27] despite presence of normal levels of phospholipase A2 [50]. Responses to both PAR-1 (SFLLRN) and PAR-4 (GYPGKF) peptide agonists are impaired in this patient demonstrating that  $G_{\alpha q}$  mediates the responses to both agonists. Genetic studies show that the  $G_{\alpha q}$  coding sequence in this patient is intact and the  $G_{\alpha q}$  mRNA levels are decreased in platelets but not neutrophils, which have normal responses and  $G_{\alpha q}$  mRNA and protein [52]. The findings in this patient with respect to platelet function have been corroborated by essentially identical abnormal responses in the  $G_{\alpha q}$ -deficient 'knock-out' mice [53].

### Defects in phospholipase C activation, calcium mobilization, pleckstrin phosphorylation

Several patients have been described with a relatively mild bleeding diathesis, and impaired aggregation and dense granule secretion, even though their platelets have normal granule stores and synthesize substantial amounts of TxA2 [54–56]. These heterogeneous patients have abnormalities in aggregation and secretion particularly in response to weaker agonists (ADP,

epinephrine, PAF); but not to relatively stronger agonists such as arachidonate and high concentrations of collagen. Such patients appear far more common than those with the storage pool deficiency or defects in TxA2 synthesis. Lages and Weiss [54] have described eight such patients who had decreased initial rates and extents of aggregation in response to ADP, epinephrine, and U44069; they postulated defects in early platelet activation events to explain the abnormal responses. They subsequently demonstrated a defect in phosphatidylinositol hydrolysis and phosphatidic acid formation [57], and in pleckstrin phosphorylation [58] in one patient.

Koike *et al.* [55] described platelet dysfunction in 12 patients with the behavioral disorder attention deficit disorder (ADD) and a mild bleeding disorder characterized predominantly by easy bruising. Platelet aggregation and  $^{14}\text{C}$ -serotonin secretion responses in PRP to ADP, epinephrine, and collagen, revealed only minor abnormalities. In contrast, the aggregation responses of gel-filtered platelets to a divalent cationophore A23187 were markedly impaired, and dense granule and acid hydrolase secretion (but not  $\alpha$ -granule secretion) was decreased in response to A23187 and thrombin at low concentrations. The platelet granule constituents were normal, thereby excluding a SPD, and platelet TxA2 production was intact. The ADD patients and those indicated above [54,56] attest to the existence of a group of patients who have platelet dysfunction despite the presence of normal granule stores and normal TxA2 synthesis; such patients have been referred to as having primary secretion defects [59].

Rise in cytoplasmic ionized  $\text{Ca}^{2+}$  concentration is an early response to platelet stimulation. Attention has therefore been focused on this process to explain the impaired platelet aggregation and secretion. In several patients, defects in calcium mobilization have been proposed based on impaired platelet responses to the calcium ionophore (A23187); however, this evidence is indirect, at best. Direct evidence has been provided that some of these patients indeed, have impaired  $\text{Ca}^{2+}$  mobilization upon platelet activation [28,51,56]. Detailed studies in two related patients [28] with impaired aggregation and secretion responses revealed that resting cytoplasmic  $\text{Ca}^{2+}$  concentration was normal, but the peak  $\text{Ca}^{2+}$  concentrations following activation with ADP, collagen, PAF or thrombin were diminished, with decreases in the release of  $\text{Ca}^{2+}$  from intracellular stores and the influx of extracellular  $\text{Ca}^{2+}$  [51]. Formation of InsP3, the key intracellular mediator of  $\text{Ca}^{2+}$  release, as well as diacylglycerol formation and pleckstrin phosphorylation, were diminished upon platelet activation [60], indicating a defect in PLC activation. Human platelets contain at least seven PLC isozymes in the quantitative order PLC- $\gamma 2$  > PLC- $\beta 2$  > PLC- $\beta 3$  > PLC- $\beta 1$  > PLC- $\gamma 1$  > PLC- $\delta 1$  > PLC- $\beta 4$  [61]. Studies in one of these patients with impaired PLC activation revealed a selective decrease in PLC- $\beta 2$  isozyme with normal levels of other PLC isoforms [61]. Genetic studies show that the decreased platelet PLC- $\beta 2$  protein levels are associated with a normal coding sequence but with diminished PLC- $\beta 2$  mRNA levels in platelets but not neutrophils, suggesting a hematopoietic lineage-specific defect in gene regulation [62]. PLC- $\beta$

isozymes are activated by G-protein-mediated pathways while PLC- $\gamma$  isozymes are activated by tyrosine kinase-dependent mechanisms [63]; the functional relative roles of the various PLC isozymes in human platelet responses remains largely unknown or the evidence is indirect. The platelet dysfunction in this patient directly validates the importance of PLC- $\beta$ 2, the predominant G-protein linked PLC isozyme in platelets in hemostasis. In line with the above findings in platelets, agonist-induced calcium mobilization is diminished in neutrophils of knockout mice deficient in PLC- $\beta$ 2 [64].

Evidence for abnormalities in signal transduction pathways in patients with diminished platelet aggregation and secretion responses has also come from other studies. Defects in phosphatidylinositol metabolism and protein phosphorylation have been described in such patients [7,57,58,65–67]. Holmsen *et al.* [65] described a patient with abnormalities in platelet aggregation and dense granule secretion who had impaired phosphoinositide hydrolysis and release of free arachidonic acid from phospholipids on thrombin activation. This patient had significant reduction in membrane GPIIb and GPIIIa as well; however, no studies were performed on  $\text{Ca}^{2+}$  mobilization or InsP3 production. In another patient the impaired platelet responses and diminished phosphoinositide metabolism have been attributed to abnormal membrane phospholipid composition [66]. Fuse *et al.* [7] have reported a patient with a mild bleeding disorder whose platelets had impaired aggregation, secretion, InsP3 formation, and  $\text{Ca}^{2+}$  mobilization in response to a TxA2 mimetic (STA2) associated with normal TxA2 formation. Interestingly, GTPase activity upon activation with STA2 was also impaired, leading to the conclusion that the platelets had an abnormality in coupling between TxA2 receptors and PLC. In the patient described by Mitsui [67], the abnormal platelet aggregation was associated with decreased TxA2-induced InsP3 formation but with normal GTPase activity and normal platelet TxA2 receptors (including their cDNA sequence), suggesting that the abnormality in PLC activity was downstream of the surface receptor. In an analysis of five patients with absent aggregation in response to TxA2 (but with abnormal responses to ADP and collagen), Fuse *et al.* [10] found evidence for a defect in the TxA2 receptor in three patients; in the other two patients TxA2-induced GTPase activity, InsP3 formation and  $\text{Ca}^{2+}$  mobilization were normal, suggesting a primary abnormality distal to the TxA2 receptor and unrelated to PLC activation. Overall, these patients provide evidence for aberrations in the signal transduction pathways in patients with diminished platelet aggregation and secretion.

Both protein kinase C-induced pleckstrin phosphorylation and cytoplasmic  $\text{Ca}^{2+}$  mobilization play a major role in secretion on activation [68]. Yang *et al.* [56] have summarized detailed studies on these responses in eight patients with abnormal aggregation and secretion in response to several different surface receptor-mediated agonists. Receptor-mediated  $\text{Ca}^{2+}$  mobilization and/or pleckstrin phosphorylation were abnormal in seven of the eight patients. It was postulated that combined platelet activation with a direct PKC activator DIC8 (1,2, dioctonoyl-*sn*-glycerol) and ionophore A23187,

which possibly bypass the receptors and two major intracellular mediators (InsP3, diacylglycerol), may induce normal dense granule secretion in these patients with diminished secretion on activation with receptor-mediated agonists. Platelet activation with a combination of ADP with DIC8 or A23187 improved secretion in four patients. However, combination of DIC8 and A23187-induced normal secretion in PRP in all patients. These studies indicate that the ultimate process of exocytosis or secretion *per se* is intact in these patients and impaired secretion results from abnormalities in early signaling events.

Protein phosphorylation by tyrosine kinases (members of the Src-kinase family, focal adhesion kinase (FAK) family, pp72syk, and Janus (JAK) kinase family) are important in platelet signaling events [69]. In thrombasthenia [70,71] and the Scott syndrome [72], tyrosine phosphorylation of several proteins is impaired on platelet activation. In these disorders, this defect is probably secondary to the primary abnormality in the GPIIb–IIIa complex and in phospholipid scrambling, respectively [69,71,72]. Interestingly, in patients with the thrombocytopenia with absent radii (TAR) syndrome, thrombopoietin-induced tyrosine phosphorylation has been reported to be markedly abnormal [73].

#### Signal transduction defects and activation of GPIIb–IIIa

Platelet activation induced conformational change in the GPIIb–IIIa complex and fibrinogen-binding to platelets is an inside-out signal transduction-dependent process. Therefore, it is likely that abnormalities in signaling mechanisms may impair activation of GPIIb–IIIa, a prerequisite for aggregation. This is supported by the report [74] of a patient with markedly abnormal platelet aggregation and receptor activated pleckstrin phosphorylation who had decreased activation of the platelet GPIIb–IIIa complexes despite the presence of normal number of these platelet receptors with intact ligand (fibrinogen) binding capacity. The implication is that defects in upstream signaling events that modulate activation of the GPIIb–IIIa complex may manifest with decreased platelet aggregation. A similar abnormality in the receptor activation of GPIIb–IIIa has also been observed in the patient with G $\alpha$ q deficiency [50] attesting to the role of G $\alpha$ q-mediated signaling mechanisms in modulating GPIIb–IIIa (Fig. 1). Such a defect in GPIIb–IIIa activation secondary to abnormalities in upstream signaling events may be a more common mechanism for blunted aggregation (particularly primary wave) than specific defects in the GPIIb–IIIa complex *per se* [75], and may explain the diminished initial aggregation responses noted by Lages and Weiss [54] in several patients.

#### Abnormalities in platelet arachidonic acid pathways and thromboxane A2 production

Platelet activation results in the liberation of arachidonic acid from phospholipids and its subsequent conversion to TxA2, which forms an important positive feedback enhancing the activation process. In the absence of TxA2 synthesis, dense granule secretion is decreased following stimulation of PRP

with ADP, epinephrine, and low concentrations of collagen and thrombin. In general, most patients with defects in the platelet arachidonic acid pathways have had mild to moderate bleeding manifestations.

### Defects in the liberation of arachidonic acid from phospholipids

The initial and rate-limiting step in TxA<sub>2</sub> synthesis is mobilization of free arachidonic acid from membrane-bound phospholipids by PLA<sub>2</sub>, a Ca<sup>2+</sup>-dependent enzyme. Four patients have been described [27] with abnormal aggregation and secretion associated with impaired liberation of arachidonic acid during platelet stimulation. Platelet TxA<sub>2</sub> production was diminished during stimulation with ADP and thrombin but was normal with free arachidonic acid. In <sup>3</sup>H-arachidonic acid labeled platelets, thrombin-induced mobilization of free arachidonic acid from phospholipids was impaired in these patients. Subsequent studies in one of the patients showed that the platelet PLA<sub>2</sub> levels (both membrane and cytosolic) were normal but agonist-induced Ca<sup>2+</sup> mobilization [51], G-protein activation and immunological Gaq levels [50] were decreased. The impaired arachidonate liberation in this patient is probably secondary to the defect in Ca<sup>2+</sup> mobilization, preventing full expression of PLA<sub>2</sub> activity. Other reports have also documented patients with an impaired release of arachidonic acid [65,76,77]. In one study [65], the platelets were also deficient in GPIIb-IIIa, while in another [77], the patient platelets had SPD in addition to diminished platelet uptake of <sup>14</sup>C-arachidonic acid and PLA<sub>2</sub> activity.

### Deficiencies of cyclooxygenase and thromboxane synthase

Several patients have been reported with a platelet cyclooxygenase deficiency, a mild bleeding disorder, and impaired platelet aggregation responses [78–84]. The patient described by Pareti *et al.* [81] had impaired PGI<sub>2</sub> production as well, and this patient with defects in both platelet and vessel wall cyclooxygenase had predominantly bleeding symptoms and not thrombotic events. The patient reported by Rak and Boda [82] had progressive arteriosclerosis as evidenced by cerebrovascular and cardiac and events. Using a radioimmunoassay, Roth and Machuga [83] found normal cyclooxygenase levels in five of six patients suspected to have a deficiency, suggesting that these patients may have a functionally abnormal molecule. More recently, three patients have been described [84] with impaired platelet responses and markedly decreased cyclooxygenase activity; using specific antibodies, the authors demonstrated decreased platelet cyclooxygenase-1 levels in two patients and normal levels in the third; levels of thromboxane synthase were normal in all three. Thus, platelet cyclooxygenase deficiency is manifested either by undetectable enzyme protein levels (type 1) or as an antigenically detectable but functionally abnormal molecule (type 2) [84].

Thromboxane synthase deficiency has been described in two patients [85,86]. Another patient has been described [87] with

bleeding manifestations, whose platelets had impaired aggregation, dense granule secretion and TxA<sub>2</sub> production upon activation. Liberation of arachidonic acid from phospholipids was normal; TxA<sub>2</sub> synthesis was markedly diminished during stimulation of PRP with thrombin, but substantial TxA<sub>2</sub> production was noted on activation of patient's platelets suspended in a buffer containing no albumin. These findings suggest that the platelets had diminished levels of enzyme activity which could express itself adequately only in the absence of albumin, which binds free arachidonic acid avidly. Although the specific enzyme defect is unknown, these studies reflect the modulating role of albumin on platelet arachidonate metabolism.

### Defects in cytoskeletal assembly

The Wiskott–Aldrich syndrome (WAS) is an X-linked inherited disorder affecting T-lymphocytes and platelets, and characterized by thrombocytopenia, small platelets with decreased survival, eczema, and immunodeficiency. Several platelet abnormalities have been reported including dense granule SPD, deficiencies of GPIb and GPIa, impaired aggregation responses and abnormalities in platelet energy metabolism [88]. WAS and the related X-linked thrombocytopenia (XLT) arise from mutations of the X-chromosome gene (location Xp11.22) called WASP which encodes a novel intracellular proline-rich 53-kDa protein of 502 amino acids [88,89]. This multifaceted WASP protein appears to constitute a link between the cytoskeleton and signal transduction pathways. WASP has been shown to bind to p47nck, a SH<sub>3</sub>-containing adapter protein, and to active GTP-complexed form of Cdc42, a member of the small GTP-hydrolyzing proteins (GTPases), which is involved in actin remodeling [88,90]. WASP has an actin-binding region at carboxyl end, and an amino terminal pleckstrin homology (PH) domain which plays a role in the binding of the protein to phospholipids (PIP<sub>2</sub>). Overall, WASP appears to be a key regulator of actin polymerization and cytoskeletal assembly leading to the unifying concept of WAS as a cytoskeletal disease [88,90]. In terms of therapy, patients with WAS respond to splenectomy and have been successfully managed with bone marrow transplantation [88,91].

### Therapy of patients with congenital platelet function disorders

Platelet transfusions and 1-desamino-8-D-arginine vasopressin (DDAVP) are the main therapy of patients with inherited platelet defects. The role of recombinant factor (F)VIIa remains promising but needs to be defined. Because of the wide disparity in bleeding manifestations, therapeutic approaches need to be individualized. Platelet transfusions are effective in controlling the bleeding manifestations but come with potential risks associated with blood products, including alloimmunization. A viable alternative to platelet transfusions is intravenous administration of DDAVP which shortens the bleeding time in a substantial number of patients with platelet function defects [92–94]. This response appears to be dependent on the defects

leading to the platelet dysfunction [92–94]. Most patients with thrombasthenia have not responded to DDAVP infusion with a shortening of the bleeding time [92–94]. Responses in patients with SPD have been variable with a shortening of the bleeding time in some patients [94–96] but not others [92,93]. A substantial number of patients with defective aggregation responses but with normal dense granule stores and TxA<sub>2</sub> production appear to shorten their bleeding times after DDAVP infusion [93]. In uncontrolled studies it has been feasible to manage selected patients with congenital platelet defects undergoing surgical procedures with DDAVP alone [92,93]. However, this approach needs to be individualized based on the nature of the surgery and the intensity of bleeding symptoms, and platelet transfusions need to be readily available for use in the event excess hemorrhage. The abnormal *in vitro* platelet aggregation or secretion responses in patients with platelet defects are not corrected by DDAVP [93]. Either an enhancement of aggregation responses by DDAVP or a direct stimulatory effect has been noted in some studies [97] but not others [98,99]. Although allogeneic bone marrow transplantation has been successfully performed with complete correction in patients with thrombasthenia [100] and the Wiskott–Aldrich syndrome [88,91], such a drastic therapy is rarely required in patients with congenital platelet function disorders.

### Future directions

Patients with a familial bleeding diathesis and abnormalities in agonist-mediated platelet aggregation and secretion are not uncommon. Yet in the vast majority of these patients the underlying molecular mechanisms leading to the platelet dysfunction are unknown, despite tremendous recent advances in our knowledge of the molecular events in platelets. The well-characterized and generally thought of entities such as thrombasthenia, the Bernard–Soulier syndrome, and storage pool deficiencies are rare or uncommon in the overall group of patients with familial platelet dysfunction, although there are geographic areas with a recognized increased prevalence of these entities. Evidence is now available for the existence of patients with specific abnormalities in the signaling events that regulate the end-responses on platelet activation, such as aggregation and secretion. The larger group of currently poorly characterized patients lumped into such categories as ‘primary secretion or activation defects’ represents an untapped reservoir of valuable new information into signaling mechanisms. There is a pressing need for concerted studies with state-of-the-art techniques to unravel the aberrant pathways in these patients.

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