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Critical Reviews in Oncology/Hematology 49 (2004) 1–35

Critical Reviews in Oncology Hematology

www.elsevier.com/locate/critrevonc

Inherited bleeding disorders: disorders of platelet adhesion and aggregation

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Accepted 9 May 2003

Contents

Abstract

Platelet aggregation at sites of vascular injury is essential for the formation of the primary haemostatic plug. The mechanism of platelet aggregation under conditions of physiological flow is a complex multistep process, which requires the synergistic action of several different

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doi:10.1016/S1040-8428(03)00117-3

platelet receptors. Platelet interaction with collagen at sites of damage to the vascular endothelium involves adhesion, activation, secretion of platelet granular contents and finally aggregation. Other agonists other than collagen, such as fibrinogen, vWF and soluble agonists released from activated platelets (thromboxane A_2 (TXA_2) and ADP) are involved in platelet aggregation. Platelets express a variety of receptors including GP Ib–IX–V, GP VI, GP Ia–IIa and GP IIb–IIIa. One aspect of this complexity of function is the variety of inherited defects of platelet function. Hereditary disorders of platelet adhesion are Bernard–Soulier syndrome and von Willebrand disease. Glanzmann thrombasthenia is an inherited disorder of platelet aggregation. The application of molecular biology to the study of platelet disorders has identified defects in other collagen receptors, ADP receptors and TXA_2 receptors. Defects affecting TXA_2 production, the generation of procoagulant activity and secretion from dense bodies and α-granules are also encountered. Other rare diseases, Chediak–Higashi, Hermansky–Pudlak and Wiskott–Aldrich syndrome also affect platelet storage granules. In this article, recent advances in the understanding of platelet function and knowledge of inherited disorders that affect platelet adhesion and aggregation is reviewed. As progress advances towards individualisation of therapy the phenotypic bleeding tendency of each patient becomes relevant.

Keywords: Inherited disorders of platelet function

1. Introduction

One definition of haemostasis is the prevention of spontaneous bleeding and the control of traumatic haemorrhage. Clinical bleeding results from a disturbance in the balance between a complex network of procoagulant and anticoagulant factors. This network involves interaction between three forces, first described by Virchow, blood (with soluble and cellular components), the blood vessel and blood flow. Normal haemostasis which is a coordinated sequence of cellular and biochemical reactions results in the formation of a platelet/fibrin aggregate. Platelets have been described as the most important cell involved in physiological haemostasis. Detailed knowledge of the haemostatic mechanism may allow a greater understanding of the pathophysiology of bleeding disorders. Platelet physiology is reviewed prior to a review of the current understanding of inherited disorders of platelet function.

2. Haemostasis

2.1. Platelets

2.1.1. Structure

Platelets, under normal circumstances, circulate in close contact to the endothelial cell lining of the vessel wall, and respond to vascular damage by adhering to subendothelial structures. This is the first step in the haemostatic plug formation. Platelet spreading follows platelet adhesion and activation, which results in secretion from platelet organelles and platelet-to-platelet interaction, called aggregation. Platelet activation also results in an increased expression of the procoagulant aminophospholipid surface to accelerate coagulation and form fibrin, which stabilises the platelet aggregate.

Resting platelets circulate as discoid anuclear cells that originated from megakaryocytes in the bone marrow. The platelet is surrounded by a typical bilamellar plasma membrane that extends through a canalicular system into the cytoplasm to greatly increase the surface area of the platelet. The equivalent of the smooth endoplasmic reticulum in platelets is the dense tubular system, where calcium is sequestered and the enzymes involved in prostaglandin synthesis are localised. The dense tubular system lies in close contact to the open canalicular system forming a membrane complex.

Of the organelles dispersed in the cytoplasm, mitochondria, glycogen particles, and lysosomes, the α granules and dense granules are the platelet specific storage granules. The α granules contain proteins such as platelet factor 4, β thromboglobulin, platelet derived growth factor, fibrinogen, fibronectin, thrombospondin, plasminogen activator inhibitor I and von Willebrand Factor (vWF). Dense bodies are rich in serotonin, adenosine diphosphate (ADP) and calcium. Platelets also produce and secrete pharmacologically active substances such as thromboxane and platelet activating factor [1].

The platelet cytoskeleton consists of a network of long actin filaments that are cross-linked by a variety of accessory proteins. Likewise, the membrane cytoskeleton as seen by thin-section electron microscopy appears to consist of a latticework of short cross-linked actin filaments that coats the inner surface of the lipid bilayer. The major function of the platelet cytoskeleton is to regulate the shape of the resting platelet and carry out contractile events such as the secretion of granules and retraction of clots by activated cells. The membrane skeleton interacts with the cytoplasmic domain of the transmembrane receptors and, in the current model, is thought to recruit signalling molecules. The activation induced reorganisation of the cytoskeleton is dynamic. The platelet extends filopodia, retracts them and extends filopodia in new directions. Examination of activated cells in suspension shows an increase in filamentous actin from 30–40 to 60–70%. The organelles are contracted towards the platelet centre and fuse with the membranes of the surface connected canalicular system. The contents of the secretory organelles are then extruded [2].

Platelets can be activated by a variety of physiological agents, soluble agonists (thrombin, ADP, epinephrine, thromboxane A_2 (TXA_2)) and adhesive proteins immobilised within the subendothelial matrix (collagen, vWF). One of the relevant issues in platelet physiology is to

Table 1
Platelet receptors

Receptor	Structure	Function	Second messenger
IIb–IIIa	$\alpha_{IIb}\beta_3$	Receptor for fibrinogen, vWF, fibronectin, vitronectin and thrombospondin	Initiates 'outside in' signals. Involves activation of intracellular kinases and elevation of intracellular calcium levels
Ia–IIa	$\alpha_2\beta_1$	Receptor for collagen	Predominantly an adhesion receptor
Ib–IX–V	Complex of four gene products	Receptor for insoluble vWF	As yet unknown. Cytoplasmic domain is attached to actin binding protein and 14-3-3ζ. Several tyrosine kinases and phosphatidyl kinases may be implicated [2]
VI	Nonintegrin receptor	Receptor for collagen	Cytoplasmic domain is attached to the Fc receptor γ chain [21]. Other second messengers implicated are the tyrosine kinase *Syk*, phospholipase Cγ2 [22], calcium and protein kinase C [23,24]

understand how platelet–vessel wall and platelet–platelet interactions are initiated in response to vascular injury and to what extent exogenous agonists are required to modulate these events.

2.1.2. Adhesion and activation receptors

Platelet adhesion, activation and aggregation are regulated by specific glycoproteins on the platelet cell surface. The largest glycoprotein is designated I, the smallest IX. The letters a and b were added when electrophoretic techniques allowed resolution of single bands into two separate bands. Integrins are heterodimers of α and β subunits. Platelets contain β_1 integrins $\alpha_2\beta_1$ (glycoprotein Ia–IIa, collagen receptor), $\alpha_5\beta_1$ (fibronectin receptor), and $\alpha_6\beta_1$ (laminin receptor). The β_3 integrins in platelets are $\alpha_{IIb}\beta_3$ (glycoprotein IIb–IIIa) and $\alpha_v\beta_3$, which share ligands such as fibrinogen, vWF, vitronectin and fibronectin (Table 1) [3,4].

The GP Ib–IX–V complex mediates the initial deposition of platelets on the subendothelium. This involves an interaction between the GP Ib–IX–V complex and vWF. vWF mediates the adhesion of platelets to sites of injury by forming a bridge between components of the subendothelium and the GP Ib–IX–V complex. vWF is a multimeric protein synthesised by endothelial cells (throughout the body) and megakaryocytes. It is stored in the Weibel–Palade bodies of the endothelial cells and α granules of the platelets and secreted into the plasma and extracellular matrix. The gene encoding vWF is approximately 180 kb in length and contains 52 exons and has been localised to chromosome 12 [5]. The primary translation product consists of 2813 amino acids and includes a signal peptide of 22 residues, a large propeptide of 741 residues and a mature subunit of 2050 residues. The domains of the protein are arranged in the sequence D1-D2-D′-D3-A1-A2-A3-D4-B1-B2-B3-C1-C2-CK. In the endoplasmic reticulum pro-vWF subunits dimerise through disulphide bonds, and form 'cystine knot' motifs, near the carboxyl termini. Pro-vWF dimers are transported to the Golgi apparatus where they form additional head-to-head disulphide bonds between the D3 domains (D1–D2 domains are shown to be obligatory for multimerisation) [6,7] near the amino-termini of the subunits yielding multimers that are $>10^4$ kDa in molecular weight. The cleavage of the propeptide site is encoded within exon 18 between aminoacid 741 of the propeptide and aminoacid 1 of the mature subunit [8]. The prosequence is composed of two homologous D1 and D2 domains, which contain two consensus sequences that are similar to those of the active site of disulphide isomerases that catalyse thiol protein disulphide interchange [6]. It is likely that the D3 domain contains the sulphydryl groups, which are involved in both intra and intermolecular disulphide bridges. In contrast, no interchain disulphide bonds have been localised to the D′ domain, which is the beginning of the mature vWF [9,10]. The vWF propeptide also targets vWF to secretory granules [11]. Transfection studies also suggest that vWF has a chaperone function and can alter FVIII intracellular trafficking causing it to colocalise with vWF in Weibel–Palade bodies [12]. The larger vWF multimers are more effective in promoting platelet adhesion in flowing blood. Recent studies show that thrombospondin-1 (disulphide bond reductase) secreted from endothelial cells reduces the average multimer size of vWF and, therefore, the haemostatic activity of vWF [13]. The A1 domain corresponds to residues 497–716 in the mature vWF, and has disulphide bridges at Cys509 and Cys695, which join the N- and C-termini. The A2 domain spans residues 717–909 and does not contain a disulphide bridge between the N- and C-termini (although there is a bridge between Cys906 and Cys907). The A3 domain spans residues 910–1111 and its termini are joined by disulphide bridges between Cys923 and Cys1109 [14].

Multimeric normal vWF does not bind appreciably to platelets in the circulation. Once vWF is immobilised in the extracellular matrix, however, platelets bind to it. Binding of vWF to GP Ib–IX–V complex is induced in vitro by exogenous modulators, the antibiotic ristocetin [15] and the snake venom protein botrocetin [16]. These phenomena suggest that conformational changes induced by surface adsorption regulate vWF binding to platelets. There is experimental evidence that the vWF molecule at high shear stress appears as an elongated filament rather than as a loosely coiled structure seen under static or low shear stress conditions [17]. vWF binds to several collagens in vitro including types I, II, III, IV, V and VI [18–20]. The binding sites for vWF in the subendothelium remain unclear. It has been shown that in

the subendothelium vWF co localises with collagen type VI and that purified vWF also binds to isolated collagen type VI [25,26]. Other studies have shown that vWF binds to isolated fibrillar collagen types I and III [27,28]. The various results suggest that one or more subendothelial collagens may be physiological ligands for vWF and that other connective tissue components may be important as well [29]. Binding sites for fibrillar collagen have been identified within vWF domain A3. Using the A3 deletion mutant it was demonstrated that the A3 domain in the vWF contains the major collagen type III domain binding site [30]. Using blocking antibodies to vWF domains A1 and A3, it was shown that to achieve maximal efficiency of vWF in initiating platelet binding to collagen VI under conditions of high shear, both A1 and A3 domains of vWF are necessary [31]. Monoclonal antibody to vWF domain A3 has been shown to inhibit vWF-collagen binding in vitro [32]. Two groups have recently reported the crystal structure of the A3 domain [33,34].

It is well established that the binding site for the GP Ib–IX–V lies within the A1 domain of the vWF [35,36]. The A1 domain (amino acids 497–716) contains two cysteine residues that form an intramolecular disulphide bond (Cys509–Cys695). The amino acid sequence residing between the two cysteines adopts a globular structure in which the hydrophobic β sheet forms a central core that is surrounded by amphipathic α helices. Studies with synthetic peptides have identified residues Asp514–Glu542 as one potential site involved in the binding of vWF to the GP Ib–IX–V complex [37]. Several studies with deletion or charged to alanine scanning mutagenesis have attempted to identify the amino acid residues that are critical for the binding of GP Ib–IX–V complex. A double mutant at Glu596 and Lys599 has been reported to impair vWF binding to GP Ib–IX–V [38,39]. Crystallographic structures were determined recently for two forms of the vWF A1 domain [40,41]. Six mutations located on the front and upper anterior face of the folded vWF A1 domain, spanning amino acid residues 524, 561, 563, 594/596, 604, 608 showed reduced activity in flow dependent platelet adhesion assays [42]. Further studies also showed that substitutions at Tyr565, Glu596, Lys599 and Gly561 in A1 domain fragments decreased their ability to mediate platelet adhesion under flow [43]. Studies with mutant vWF protein suggest that the amino acid residues required for botrocetin induced binding to GP Ib are distinct from those required for ristocetin induced binding and are within the A1 domain [44]. This was confirmed by studies with monoclonal antibodies to vWF and synthetic peptides derived from the sequence of vWF [45]. The site of interaction for IIb–IIIa the second platelet receptor that binds to vWF is located at residues 1744–1747 (Arg–Gly–Asp–Ser) in the carboxyl-terminal of the C1 domain [46]. The binding of vWF to FVIII is required to stabilise FVIII in the circulation. The binding site for FVIII is within the 1–272 residues (encoded by exons 18–23) of the amino terminal of the mature vWF subunit [47]. The epitopes of anti-vWF monoclonal antibodies, which block FVIII binding to vWF, have been mapped to amino acid residues in positions 2–53 [48], 51–60 [49] and 78–96 [50].

GP Ib–IX–V complex is constitutively expressed on the platelet surface (≈25 000 copies/platelet). It is made up of four distinct gene products: GP Ibα (≈135 kDa, coded on chromosome 17) disulphide bonded to GP Ibβ (≈25 kDa, coded on chromosome 22) and the non-covalently associated subunits GP IX (≈22 kDa) and GP V (≈82 kDa). Genes encoding the latter are found on chromosome 3. GP V is non-covalently associated with GP Ibα and appears to link two GP Ib–IX complexes together [51]. GP IX can form partial complexes with GP Ibβ but associates loosely if at all with GP Ibα [52]. All are members of the leucine rich protein family and contain ≈24 amino acid leucine rich sequences, which occur singly or in tandem repeats, in their extracellular domains. The seven tandem leucine rich sequences of GP Ibα are flanked by conserved disulphide loop structures at both the N- and C-termini of the repeats. Downstream of the C-termini of the repeats are three sulphated tyrosine residues [53]. Though a number of studies have localised the binding of vWF to the N-terminal domain of GP Ibα, the role of the individual domains within the His1–Leu275 residues, which include the leucine rich residues and their flanking sequences remains equivocal [54]. Expression of the recombinant polypeptides making up the receptor complex in mammalian cells showed that all subunits of the GP Ib–IX complex were required for stable surface expression. The role for GP V was less well defined [55,56]. Studies on the crystal structures of amino terminal of GP Ibα (residues 1–290 with the leucine repeats) and vWF A1 domain, show that the GP Ibα wraps around the A1 domain, with two points of contact. The carboxy-terminal binds near the top of the A1 domain, and the amino-terminal near the base [57].

Pioneering studies in several laboratories led to the proposal that the integrin $\alpha_2\beta_1$ (GP Ia–IIa) was the platelet collagen receptor. This receptor was first recognised in a patient with congenital bleeding disorder and a total lack of interaction with collagen [58,59]. Monoclonal antibody against GP Ia completely inhibited platelet adhesion to collagens type I–VIII and monoclonal antibody against IIa partly inhibited collagen adhesion under conditions of both stasis and flow. Binding to GP Ia–IIa is now recognised as the major interaction supporting platelet adhesion to most forms of collagen [60]. The N-terminal ≈440 amino acid of integrin α subunits contains seven sequence repeats, and it has been predicted that they fold into a β propeller domain [61]. Some α subunits also contain an inserted (I) domain, between the repeats. Fewer modelling studies have been made with integrin β subunits but the N-terminal sequence has similarities to the I domain and to a metal ion dependent adhesion site (MIDAS) domain and may be folded into a similar structure. Binding of GP Ia–IIa occurs to collagen through the α_2 I domain (homologous to the vWF A domain) which contains a trench, centred on the MIDAS motif, which binds Mg^{2+} as in other integrin I domains [62]. Recent analysis of the crystal structure of the complex with a collagen-mimetic

peptide indicated that the binding domain undergoes a dynamic structural change upon complex formation with a collagen mimetic peptide [63]. Upon agonist (thrombin, ADP and collagen related peptides) stimulation of platelets, GP Ia–IIa is activated to a form with a high affinity for soluble collagen, further suggesting the existence of different conformations of GP Ia–IIa that determine ligand affinity [64].

There is evidence that adhesion and activation of platelets by collagen is mediated through distinct receptors. Evidence for this came from the observations that a collagen based octapeptide [65] and the cross linking agent 3,3′ dithiobis(sulphosuccinimidyl)propionate [66] inhibited platelet activation by collagen but not adhesion. Studies with GP VI deficient patients whose platelets exhibited impaired activation by collagen highlighted GP VI as the receptor for collagen mediated activation [67]. Recent cloning and characterisation of the gene coding for GP VI showed that it belonged to the immunoglobulin superfamily [68,69]. Evidence for a collagen dependent pathway other than GP Ia–IIa underlying platelet activation is also provided by studies with synthetic collagens and snake venom proteins. Although collagen-like, triple helical peptides based on glycine–proline–hydroxyproline repeat sequence cannot bind GP Ia–IIa, they are potent platelet activators [70,71]. Direct evidence that platelet activation by collagen related peptides is mediated through GP VI is provided by the observation that platelet activation by collagen related peptides is lost in GP VI deficient platelets [72]. The snake venom convulxin, which binds predominantly if not exclusively to GP VI is also a powerful platelet activator [73] compared with collagen [74]. The unique properties of these specific activators offer opportunities for further exploration of the signalling mechanism of GP VI. Studies using cyanogen bromide fragments of collagen provided additional support for the dissociation of adhesion from activation as both events were mediated by different regions of the collagen molecule [75]. A further question regarding the role of GP Ia–IIa is whether the receptor is a silent adhesive receptor for collagen or whether it also generates intracellular signals that support platelet activation. Evidence for the role of GP Ia–IIa in platelet activation is provided by the use of snake toxins trimucytin and aggretin. Both toxins are platelet stimulants and it is claimed that their action is mediated through GP Ia–IIa as antibodies to this receptor prevent platelet activation by these compounds [76,77]. Monoclonal antibody raised against the GP Ia subunit was shown to partially inhibit aggregation by the GP VI selective ligand convulxin, suggesting that GP Ia–IIa and GP VI are involved in platelet activation induced by convulxin [78]. In some studies, GP Ia–IIa selective peptides do not stimulate tyrosine phosphorylation [79], though other authors suggest GP Ia–IIa interaction with collagen is accompanied by *c-Src* activation and tyrosine phosphorylation [80]. Though cross linking antibodies to GP Ia–IIa stimulate phosphorylation of *Syk* and PLCγ2, this response is dependent on the presence of FcγRII suggesting that a co-receptor in addition to GP Ia–IIa is required for these signalling events [81]. Recently it has been suggested that trimucytin may induce activation through GPVI rather than GP Ia–IIa [82].

Other platelet glycoproteins that are candidates for collagen receptors are GP IV and the recently cloned receptor p65. However, GP IV is absent in 3–5% of the Japanese population who display no major bleeding problems. Further their platelets show normal adhesion during flow, normal aggregation and normal platelet metabolic responses to collagen [83,84]. The relationship of the p65 receptor to others involved in the collagen–platelet interaction is unclear [85,86]. A platelet receptor for type III collagen, Type III collagen binding protein with a molecular weight of 68 kDa, has been recently described [87].

2.1.3. Shear stress and platelet function

Platelet adhesion occurs mostly from flowing blood and the normal haemostatic response is as a result, influenced by the flow of blood. Any explanation of platelet adhesion and aggregation needs to take into account rheological variables and go beyond observations in static conditions. Wall shear stress is the force per unit area applied by the flowing viscous blood to the vessel surface and shear rate is the local velocity gradient. The wall shear rate (per s) is described as 300–800, 500–1600, 20–200, and the wall shear stress (dyn/cm^2) 11.4–30.4, 19.0–60.8, 0.76–7.6, in large arteries, arterioles and veins [88]. In flowing blood, red blood cells tend to stream to the centre of the vessel lumen whereas platelets distribute to the lumen periphery [89].

To examine the role of GP Ib–IX–V complex under dynamic conditions (shear stress of 2–40 dyn/cm^2) initial adhesion of transfected mammalian cell lines expressing the GP Ib–IX–V complex was observed in a parallel plate flow chamber using vWF coated glass slides. In these studies it was shown that GP Ib–IX–V complex behaves like selectin receptors in its ability to mediate smooth rolling of cells and maintain surface contact. In vivo this would result in slowing down of the platelets on the blood vessel wall [90].

At high shear rates (10 800/s) soluble vWF was shown to bind to a small proportion of washed platelets, and the use of selected monoclonal antibodies against either GP Ibα or GP IIb–IIIa showed that both receptors were involved in the process [91]. Local concentrations of agonists such as epinephrine [92] and ADP [93] through their specific receptors, may enhance shear induced platelet aggregation by soluble vWF.

Other studies with platelets under flow [94] confirm that the binding between immobilised vWF and GP Ib–IX–V is reversible, the bridging interaction slowing the platelet temporarily near the injured blood vessel. In experiments in which platelets labelled with mepacrine were observed to adhere and aggregate to collagen coated surface under different shear rates, platelet adhesion to collagen coated surfaces were shown to be dependent on vWF/GP Ib–IX–V interactions especially at high shear rates. Under these flow conditions, permanent arrest of platelets required interaction

of the collagen surface with GP Ia–IIa. The function of the GP Ia–IIa receptors became more significant as platelets were subjected to increasing shear rates on surfaces with relatively sparse collagen fibrils [95]. The results suggest that GP Ia–IIa is necessary for more permanent tethering of floating platelets to the collagen fibre [96]. Moroi et al. [97] using a flow perfusion chamber showed that platelets bound to the collagen surface in a biphasic manner. In the first phase platelets stopped transiently on the surface, and in the second phase platelets formed aggregates. Antibodies to GP IIb–IIIa inhibited platelet aggregation. GP VI deficient platelets also showed defective platelet aggregation. Earlier studies on the time course of inhibition with selective antibodies support a model in which collagen interacts directly with GP Ia–IIa but with GP IIb–IIIa through intermediary adhesive proteins [98]. The tethering of platelets to vWF matrix under flow conditions results in rapid morphological conversion of platelets from flat discs to spiny spheres during surface translocation. Studies with Chinese Hamster Ovary cells transfected with GP Ib–IX and Glanzmann thrombasthenia platelets (lacking GP IIb–IIIa) show that vWF binding to GP Ib–IX was sufficient to induce actin polymerisation and cytoskeleton reorganisation [99]. Further studies show that pre-treating platelets with inhibitors of actin polymerisation dramatically increased the rate of platelet aggregation induced by vWF, suggesting a complex role for the cytoskeleton in regulating the adhesive functions of GP Ib-V-IX [100].

The mechanism of platelet adhesion, activation and aggregation can be viewed as a two step mechanism. The first step is the binding of GP Ibα and vWF. The vWF–GP Ibα interaction is characterised by a fast association and dissociation rate, supporting the movement of platelets in the direction of flow, even at high shear rates. Interaction with GP Ia–IIa and GP VI, results in platelet arrest, activation and aggregation. The precise role of GP Ia–IIa in activation is still unclear [101,102].

Glycoprotein IIb–IIIa, expressed on non-stimulated platelets is another major platelet adhesion receptor. The GP IIb consists of a heavy chain, GP IIbα with a molecular mass ≈135 kDa and a light chain GP IIbβ with a molecular weight ≈25 kDa linked by a disulphide bond. The GP IIIa subunit is a glycosylated polypeptide with a molecular weight of ≈95 kDa. The genes for both proteins are found on chromosome 17 [103]. GP IIb–IIIa is one of the most abundant platelet surface receptors (≈80 000 per platelet) [104]. The GP IIb unit is synthesised as a single-chain precursor that associates with GP IIIa within the endoplasmic reticulum, the pro GP IIb–IIIa is transported to the Golgi apparatus where GP IIb undergoes cleavage into heavy and light chains, prior to transport to the cell surface [105,106]. GP IIb–IIIa recognises at least four adhesive receptors, fibrinogen, fibronectin, vitronectin and vWF.

GP IIb–IIIa is normally present in a ‘low activation’ resting state. In this state, it does not bind soluble fibrinogen or vWF in a productive fashion. However, immobilised fibrinogen can bind to GP IIb–IIIa in an irreversible manner at wall shear rates below 600/s [107]. GP IIb–IIIa is in a resting state when it is recognised by surface bound fibrinogen. The fact that immobilised fibrinogen but not soluble fibrinogen can bind to ‘resting’ GP IIb–IIIa suggests that fibrinogen is changed following immobilisation [108]. Three potential binding sites for GP IIb–IIIa have been proposed. The α chain of fibrinogen contains Arg–Gly–Asp sequence at positions 95–97 and 572–574, and the γ chain has a unique dodecapeptide sequence (His400–Val411) at the carboxyl terminus. Measurement of fibrinogen binding to GP IIb–IIIa using fibrinogen with mutated Arg–Gly–Asp or γ chain sequence indicates a predominant role for the γ chain sequence in fibrinogen binding [109]. Other studies suggest that multiple sites are responsible for the adhesive properties of fibrinogen to intact platelets [110]. Soluble agonists, which act through their specific membrane receptors to increase the affinity of GP IIb–IIIa for vWF and fibrinogen are thrombin, ADP, epinephrine, TXA_2. The binding of immobilised vWF to GP Ib leads to a change in the ligand recognition specificity of GP IIb–IIIa such that it can mediate irreversible platelet adhesion and aggregation [111]. Recent studies [112,113] have shown that in heterologous Chinese hamster ovary cells expressing both GP IIb–IIIa and GP Ib–IX, GP IIb–IIIa is activated by signalling from GP Ib–IX by its ligand vWF. Using a static platelet adhesion assay, Nakamura et al. [114] showed that platelet adhesion to collagen can induce the activation of GP IIb–IIIa and that the activation process is mediated by collagen interaction with both receptors GP Ia–IIa and GP VI (Fig. 1).

GP IIb–IIIa supports platelet to platelet interaction, aggregation, by binding to fibrinogen [115]. Similarly, aggregation is promoted by vWF interacting with GP IIb–IIIa [119]. vWF is unique in its ability to support the initial transient phase of platelet adhesion as well as GP IIb–IIIa mediated platelet aggregation [116]. Shear (180 dyn/cm^2) induced platelet aggregation, studied in a cone and plate viscometer, was inhibited by antibodies to GP Ib or the GP IIb–IIIa complex, but was little affected by the absence of fibrinogen, again suggesting the dependence of platelet aggregation on vWF [117]. Although G proteins, intracellular calcium, protein kinases and many other proteins are involved in the expression of the activated GP IIb–IIIa complex (inside-out signalling) the exact mechanisms that control the expression of the activated GP IIb–IIIa complex are still obscure [118]. Studies suggest that changes in the actin cytoskeleton allow GP IIb–IIIa to assume the conformation required for ligand binding [120]. GP IIb–IIIa, in addition to adhesive functions, generates signals (outside–in signalling) that result in platelet aggregation and spreading [121].

A thrombus is constituted essentially of platelets and fibrin, thus its development depends on the formation of inter-platelet bonds. Shear induced binding of soluble vWF to GP Ibα results in the binding of fibrinogen to GP IIb–IIIa. The dimeric structure of fibrinogen enables it to cross-link adjacent activated platelets. In the traditional model of platelet aggregation GP IIb–IIIa was thought to have an

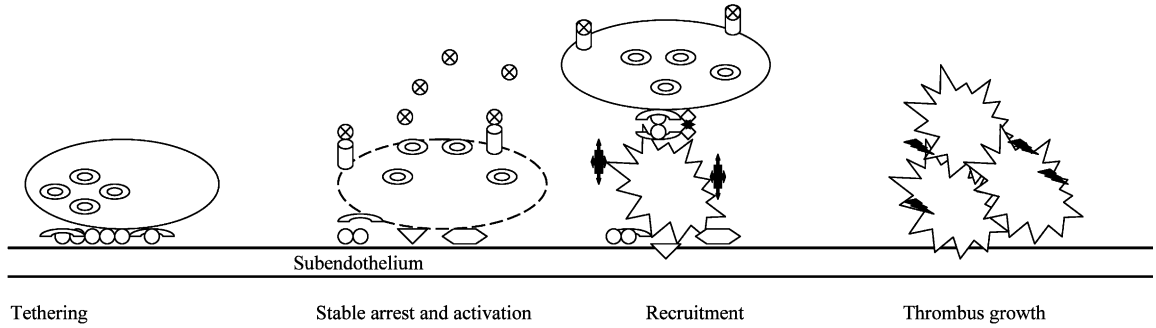

Fig. 1. Schematic representation of the mechanisms of platelet adhesion and aggregation. Collagen bound vWF tethers circulating platelets by interacting with GP Ib–IX–V. Platelet activation occurs when collagen binds to the platelet through GP Ia–IIa and GP VI. Activated platelets express a procoagulant surface as shown by the broken lines. Activated platelets release granule contents. Soluble agonists contribute to the activation of GP IIb–IIIa. VWF–GP Ib–IX–V interaction also tethers circulating platelets to the immobilised platelets. Stationary platelet-to-platelet adhesion is dependent on the activation of GP IIb–IIIa and its subsequent binding to fibrinogen. GP Ib–IX–V, vWF, platelet granules, soluble agonist ⊗, GP Ia–IIa, GP IV, soluble agonist receptor, prothrombinase or tenase complex, Fibrinogen, GP IIb–IIIa ◇, Cross linked Fibrin.

exclusive role in mediating platelet aggregation. Recent studies suggest that for a platelet suspension, at high shear rates, GP Ibα–vWF interaction may promote inter-platelet interaction regardless of activation. The newly recruited platelets become activated by locally generated agonists. Binding of soluble adhesive ligands such as fibrinogen to the membrane makes the aggregation irreversible. The interaction of GP Ibα–vWF has a fast forward rate with a high resistance to tensile stress, but is transient due to a high dissociation rate. Interactions involving GP IIb–IIIa are of limited efficacy at high velocity shearing flow because of the relatively slow forward rate. These distinct properties of the two receptors support a stable inter-platelet adhesion because the initially transient tethering created by GP Ibα–vWF interaction prolongs the time available for activation and irreversible bond formation mediated by GP IIb–IIIa [122].

In studies in which flowing blood adhered to the surface of immobilised platelets it was shown that the majority of platelets adhering to the luminal surface of the developing thrombus subsequently detach, and that the GP Ibα–vWF interaction was required for initial platelet to platelet attachment at both arterial (1800/s) and venous (150/s) rates of shear. A key finding in these studies was the role of GP Ibα–vWF in initiating inter-platelet interactions in free flowing blood. Moreover, the studies performed on platelet monolayers prepared from individuals with severe von Willebrand's disease (<1% vWF) demonstrated a role for platelet vWF in mediating platelet to platelet tethering and translocation through a specific interaction with GP Ibα [123]. Subsequent interaction of activated GP IIb–IIIa with fibrinogen allows permanent bridging across inter-platelet contacts [124]. Thus, at both early and late stages of thrombus formation, platelet adhesive mechanisms, in rapidly flowing blood, depend on multiple bonds, involving different receptors and ligands with specific functions. It has been suggested that in areas at the edge of the thrombus the shear rate increases as a function of distance from the wall because narrowing of the lumen causes increased local flow velocity for a constant volumetric flow [125].

2.1.4. Other agonists

2.1.4.1. Thromboxane. The extent of aggregation of platelets is intensified by the effects of TXA_2. TXA_2 is synthesised in platelets following the release of arachidonic acid from membrane phospholipids. Arachidonic acid is subsequently metabolised by cyclooxygenases and TXA_2 synthase. The major prostaglandin synthesised by the endothelial cells is prostacyclin-PGI_2. Thromboxane promotes the formation of haemostatic plugs by aggregating platelets and constricting blood vessels, whereas prostacyclin PGI_2 counteracts thromboxane by suppressing platelet aggregation and dilating vessels. Thromboxane and PGI_2 are extremely labile with half lives of 30 s and 3 min, respectively [126,127].

The mRNA for TXA_2 receptor subtypes TPα, and TPβ have both been shown in human platelets [128], though it is not known if both forms are translated to protein [129]. The TP receptor gene has been cloned and encodes the two subtypes of TP receptor that result from alternate splicing of the primary transcript. The subtypes possess different carboxyl terminal domains [130]. Both the TPα and TPβ subtypes mediate, through a G-protein, the stimulation of phospholipase C (PLC) and an increase in the intracellular concentrations of inositol 1,4,5-triphosphate and diacylglycerol. The formation of inositol 1,4,5-triphosphate induces an increase in the cytosolic concentration of Ca^{2+}, whereas the release of diacylglycerol activates protein kinase C. In transfected cell lines thromboxane binding to the TPα receptor activated adenyl cyclase activity while binding to the TPβ receptor inhibited it [128]. Results from previous studies showing the involvement adenyl cyclase in TXA_2 mediated pathway have been conflicting [131]. Other studies show that TXA_2 induced inhibition of adenyl cyclase depends on secretion

of granule contents and the secretion of other agonists that inhibit adenyl cyclase [130].

2.1.4.2. Adenosine diphosphate (ADP). Addition of exogenous ADP to washed human platelets results in shape change and aggregation. In addition platelets release granule contents, produce thromboxane and activate the fibrinogen receptor [132,133]. It is thought that the close platelet-to-platelet contact induced by weak agonists like ADP, triggers the formation of TXA_2 and release of granule contents, which reinforces aggregation. This effect is mostly observed when the concentration of calcium in the extracellular medium is artificially decreased [134,135]. Evidence suggests that no single receptor can mediate the full response of platelets to ADP. Based on studies with selective agonists and antagonists, human platelets display at least three distinct purinergic receptors for ADP [136–139]. The $P2X_1$ ionotropic receptor is responsible for rapid influx of ionised calcium into the cytosol [140]. The $P2Y_1$ receptor is responsible for the intracellular mobilisation of ionised calcium into the cytosol through activation of PLC, as well as shape change and aggregation. The $P2Y_{12}$ receptor is coupled to adenyl cyclase inhibition. Both the $P2Y_1$ and $P2Y_{12}$ receptors have been cloned by different investigators [141–144]. $P2Y_1$ and $P2Y_{12}$ receptors are G protein coupled receptors. Simultaneous activation of $P2Y_1$ coupled to G_q and $P2Y_{12}$ coupled to G_i is required for the complete aggregation response [137]. The availability of mice deficient in either $P2Y_1$ and $P2Y_{12}$ makes it possible to assess the relative contributions of each receptor to platelet activation in vivo [145,146]. The $P2Y_1$ receptor is thought to solely mediate shape change [147]. The $P2Y_1$ receptor is necessary to initiate ADP induced shape change and aggregation and the $P2Y_{12}$ receptor is essential for the completion and amplification of the response to ADP. Antagonism of either receptor is sufficient to inhibit aggregation, suggesting that both receptors interact to lead to the full response.

Other agonists that activate platelets by binding to G protein coupled receptors on the platelet surface are thrombin, TXA_2, and epinephrine. The latter activates platelets through the α_{2A} adrenergic receptor. Key questions that remain unanswered are the nature of the G protein pathway of ADP-induced platelet aggregation, and the features that are responsible for the different efficiencies with which different receptors couple to the various G proteins. Platelets from mice that lack the α subunit of G_q display ADP related platelet defects similar to platelets from mice that lack $P2Y_1$, they neither change shape or aggregate but retain their ability to change shape in response to other agonists, suggesting that another member of the G protein family mediates shape change in response to other agonists [148–150]. The relative abilities of each receptor for platelet activation may contribute to platelet pathology and to the clinical presentation in bleeding diathesis. It is unclear if the $P2X_1$ receptor can activate any physiological platelet responses. Recent publications suggest that $P2X_1$ evoked calcium influx not only potentiated $P2Y_1$ mediated calcium increase but also independently causes platelet shape change [151,152]. The case of a patient with a bleeding disorder which might be due to the presence of a mutated form of the $P2X_1$ receptor has been recently described [153]. Other reports suggest that the receptor $P2X_1$ is a minor player and that it may be a receptor for ATP rather than ADP [154]. CD39 the endothelial cell ecto-ADPase, is a further component responsible for limiting platelet activation, by hydrolysing ADP to AMP [155].

2.1.4.3. Thrombin. Thrombin mediates shape change and the release of granule contents. It also activates GP IIb–IIIa, which results in the binding of fibrinogen and vWF [156]. Thrombin signalling is mostly mediated by a family of G protein-coupled protease receptors (PAR). Four PAR's (PAR1, PAR2, PAR3 and PAR4) have been identified. PAR1 mRNA has been detected in human platelets using reverse transcriptase polymerase chain reaction. PAR4 mRNA was also detectable in human platelets at 10–30% of PAR1 mRNA levels. PAR3 mRNA was not detectable in human platelets and PAR2 is activated by trypsin and trypsin like proteases but not by thrombin. Flow cytometry using PAR-specific antisera confirmed that PAR1 and PAR4 are expressed on the surface of human platelets [157]. PAR1 is a member of the seven transmembrane domain family of receptors but is activated by a novel mechanism. PAR1 is activated when thrombin binds its amino terminal exodomain LDPR↓SFLLRNPNDKYEPF and cleaves at the Arg41–Ser42 peptide bond to unmask a new receptor amino terminus. The new amino terminus then serves as a tethered peptide ligand, binding intramolecularly to the body of the receptor to effect transmembrane signalling [158,159]. Studies with mutant receptors and receptor peptides revealed two domains within the receptor's amino-terminal exodomain, the cleavage recognition sequences LDPR and the 'hirudin-like' domain DKYEPF to be important for the thrombin receptor interaction. Recent X-ray crystallographic studies of thrombin co-crystallised with receptor peptides showed that the LDPR sequence can bind to the thrombin active centre and that the DKYEPF sequence can bind with the thrombin anion binding exosite [160]. Further, replacing the PAR1 thrombin cleavage site LDPR↓S with the enteropeptidase cleavage site DDDDK↓S produced a receptor that signalled to enteropeptidase and not to thrombin [161]. Par-4 is relatively insensitive to thrombin, with an EC_{50} for the protease approximately 50-fold higher than the corresponding figure for PAR-1. In addition PAR-4 does not contain a hirudin-like domain [156].

The synthetic peptide SFLLRN that mimics the first six amino acids of the new amino terminus unmasked by receptor cleavage functions as an agonist for PAR1 and activates the receptor independently of thrombin and proteolysis. The PAR1 peptide SFLLRN and the PAR4 peptides GYPGKF and GYPGQV (corresponding to the PAR4 tethered ligand) all activated human platelets. At low concentrations of thrombin (1 nM), anti-PAR1 immunoglobulin

inhibited platelet aggregation, while in contrast, anti-PAR4 immunoglobulin by itself had no effect on platelet aggregation in response to low concentrations of thrombin. These data suggest that PAR1 is the major mediator of thrombin signalling at low concentrations of thrombin. At higher concentrations of thrombin (30 nM) inhibition with anti-PAR1 immunoglobulin only slowed aggregation slightly. Inhibition of PAR4 signalling with anti-PAR4 antibody was similarly ineffective. When signalling through PAR1 and PAR4 was blocked simultaneously platelet aggregation even at high concentrations of thrombin was eliminated. The data suggest that at high concentrations of thrombin both PAR1 and PAR4 contribute to platelet activation [162]. Following activation by thrombin, PAR1 is rapidly phosphorylated and uncoupled from signalling after activation. Activated PAR1 recycles mainly to lysosomes. Recent studies with chimeric receptors suggest that the cytoplasmic tails of PAR1 specify distinct intracellular sorting patterns. In fibroblasts and endothelial cells, unactivated PAR1 appears to cycle slowly between the cell surface and the intracellular compartment such that at the steady state approximately one-half of PAR1 molecules are inside the cell and protected from thrombin cleavage. This intracellular reserve can repopulate the cell surface with naïve receptors, without new receptor synthesis, thus maintaining responsiveness to thrombin [163–165].

GP Ibα subunit also has a thrombin binding site on the extracellular domain that overlaps with the vWF binding domain, including residues 271–284, and is analogous to the thrombin binding sequence in hirudin [160]. The anion binding exosites of thrombin has been implicated as the site of thrombin–GP Ibα interaction [166]. Additionally the complex has a platelet specific thrombin substrate, GP V that is cleaved by thrombin [167]. It was shown that the platelets of GP V −/− mice with the normal GP Ib–IX receptor showed an increase in platelet response to thrombin, suggesting that GP V was an inhibitor of platelet function. Recently, Ramakrishnan et al. [168] have shown that removal of GP V, by gene targeting, permits platelet activation by protease-inactive thrombin. The data suggest that GP Ibα-bound thrombin can initiate additional signalling responses in platelets, and that GP Ib–IX–V complex functions as a thrombin receptor after it jettisons GP V chain either through thrombin cleavage or gene knockout. However, it can be argued that the removal of GP V by gene targeting, results in abnormal GP Ib–IX expression.

Development of procoagulant activity involves flip-flop of lipids within the platelet plasma membrane so that negatively charged aminophospholipids, phosphatidylserine, phosphatidylethanolamine and inositol are exposed on the outer surface of the plasma membrane. The mechanism of expression of procoagulant activity in response to platelet agonists is yet to be unravelled. Using the thrombin receptor peptide SFLLRN, it has been shown that activated PAR1 does not expose procoagulant activity [169]. Because exposure of negatively charged aminophospholipids at the platelet surface is essential for procoagulant activity, its measurement by binding of annexin V is a good method for determining the first step in the process. A role for GP Ib–thrombin interaction in procoagulant activity has been proposed on the basis that inhibition of thrombin binding to GP Ib inhibited annexin V binding to platelets [170]. GP IIb–IIIa is required as well for procoagulant expression in response to thrombin [171]. Thus thrombin plays an important role in the cross-talk between the fibrin and platelet pathways.

Thrombin also has a host of direct actions on cells. Thrombin and platelets activated by thrombin induce cultured endothelial cells, to secrete chemokines and to express adhesion molecules thereby generating signals for the recruitment and extravasation of leukocytes at the site of injury [172,173]. PAR1 seems to be the major mediator of thrombin signalling in vascular endothelial cells; most of the actions of thrombin on endothelial cells have been reproduced using PAR1 agonist peptide. Thrombin also stimulates endothelial contraction and increased permeability, further facilitating the transmigration of neutrophils to sites of vessel damage [174,175]. In fibroblasts, vascular smooth muscle and endothelial cells, thrombin stimulates increased DNA synthesis and promotes cellular proliferation [176]. In cultured cells thrombin also activates and modulates matrix metalloproteinases including progelatinase A and metalloproteinases 1, 2 and 3. These are key enzymes involved in the degradation of the basement membranes, which, along with endothelial cell migration, and proliferation, is a first step in the initiation of angiogenesis [177]. These actions suggest that thrombin acts to coordinate the haemostatic and inflammatory response and participates in the wound healing process [178].

2.1.5. Inhibitors

Nitric oxide and certain prostaglandins (PGI_2, PGD_2) are inhibitors of platelet aggregation. Nitric oxide is synthesised from L-arginine by the constitutive enzyme nitric oxide synthetase in the vascular endothelium. Nitric oxide is a vasodilator and contributes to the control of platelet aggregation. PGI_2 and PGD_2 exert their effects through specific membrane receptors to increase cAMP formation. Nitric oxide diffuses through platelet membranes to increase cGMP levels [179]. Cross talk between the cAMP and cGMP systems occurs at the phosphodiesterase level. Several groups have shown that nitric oxide donors cause small increases in platelet cAMP. Work with selective phosphodiesterase inhibitors has shown that the additional increase in cAMP is attributable to inhibition of phoshodiesterase3 by cGMP. However, under certain conditions nitric oxide donors cause an inhibitory effect on cAMP accumulation in human platelets, and this has been attributed to activation of phosphodiesterase2 [180]. It remains to be determined if cross talk between the cAMP and cGMP systems occur at the level of protein kinase activation [181,182].

2.2. Coagulation pathway

A detailed description of the coagulation pathway is beyond the scope of this paper and a brief overview is given. The reader is also referred to several journal articles, including those by the author, for detailed reviews on coagulation and fibrinolytic pathways [183–186]. The coagulation cascade provides a mechanism for converting mechanical information of tissue damage into biochemical response. Blood coagulation involves a complex series of interactions between protease zymogens, enzymes and cofactors that lead to the generation of thrombin and a fibrin clot. This complex biological system involves a number of zymogens (factors XII, XI, X, IX) and prothrombin (factor II) and procofactors (factors V and VIII) which must be converted into the corresponding enzymes and cofactors before they interact in a cascade system and transform fibrinogen to fibrin. The coagulation pathway is divided into the intrinsic pathway in which exposure of the contact factors (FXII, high molecular weight kininogen and prekallikrein) in plasma to a surface initiates coagulation and the extrinsic pathway which is initiated by exposure of the membrane bound cofactor of FVII, tissue factor (Fig. 2). It was inferred from the absence of bleeding associated with deficiencies of FXII, prekallikrein, and high molecular weight kininogen that these factors were not the primary route of FIXa generation. The observation that FVIIa–TF could activate FIX and that thrombin can activate FXI showed the pivotal role of TF in the initiation of coagulation. Some of these reactions depend on calcium ions and negatively charged phospholipids. Intrinsic tenase, composed of FVIIIa–FIXa, negatively charged phospholipids and calcium, converts FX to FXa. Thrombin is formed by an enzyme called prothrombinase composed of FXa, FVa, negatively charged phospholipids and calcium. The biological elements contributing to the phospholipid matrix are damaged vascular tissue, activated platelets and inflammatory cells. The phospholipid surface on the surface of activated platelets is important as it directs fibrin formation to the site of damage and enforces the sealing effect of the platelet plug. Thrombin breaks down fibrinogen, with the release of fibrinopeptides to form soluble fibrin, which leads to the formation of FXIII, mediated cross linked fibrin. Thrombin is the main effector protease of the coagulation cascade. The coagulation system is tightly regulated by the anticoagulant systems such as antithrombin, protein S, activated protein C and the recently described tissue factor pathway inhibitor.

The fibrinolytic system is activated in response to thrombus formation. The proenzyme plasminogen is converted to plasmin by plasminogen activators. Plasmis proteolytically degrades fibrin within the clot. In vivo plasminogen activation is stimulated by tissue type and urokinase-type plasminogen activators. The main fibrinolytic reaction involves inhibition of fibrinolysis by plasminogen-activator inhibitor type 1 (PAI-1) and α_2-antiplasmin. PAI-1 is an inhibitor of plasminogen activation and α_2-antiplasmin neutralises plasmin activity [187].

The platelet surface serves as an assembly site for the components of the plasminogen activator system. Plasminogen and tissue type plasminogen activator and urokinase-type plasminogen activator bind to the platelet surface. Platelets enhance the activation of plasminogen by tissue plasminogen activator, by lowering the Km. As a consequence, the catalytic efficiency of plasminogen activation is increased [188]. PAI-1 is synthesised by endothelial cells and PAI-1 present in blood is also contained in platelet α granules [189]. α_2-antiplasmin is synthesised and secreted by hepatocytes and is also present in α granules [190]. In vitro studies show that inhibition by plasmin of platelets can occur

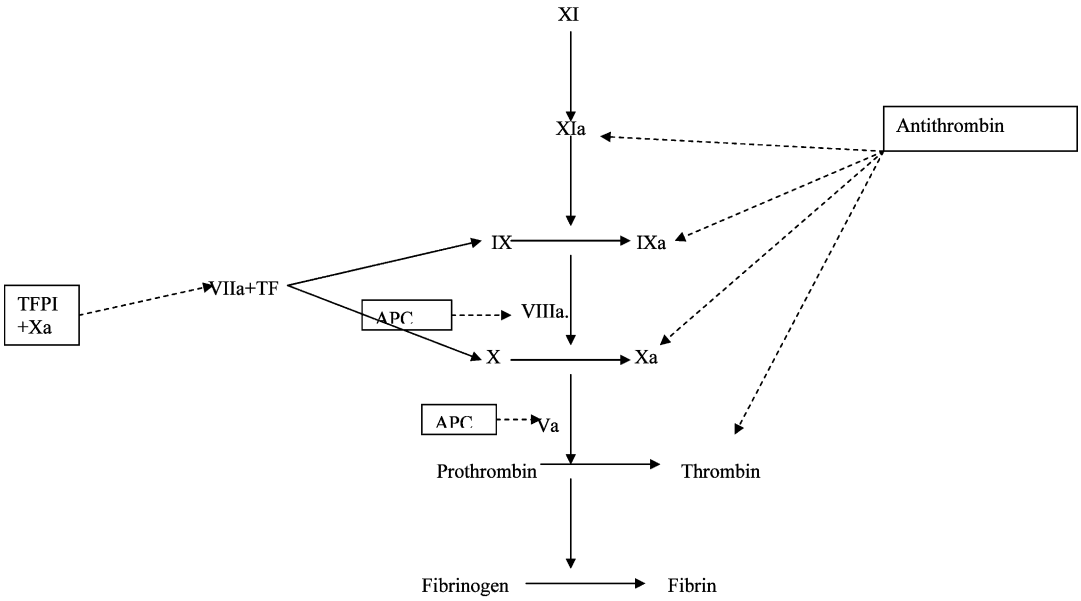

Fig. 2. Blood coagulation pathway. Broken arrows show regulatory pathways. Tissue Factor Pathway Inhibitor (TFPI). Activated Protein C (APC).

Table 2
Clinical presentation of bleeding disorders

Clinical signs	Disorders of coagulation	Disorders of platelets or vessels
Petechiae	Rare	Characteristic
Superficial ecchymoses	Common: large and solitary	Characteristic: small and multiple
Bleeding from superficial cuts and bruises	Minimal	Persistent: often profuse
Delayed bleeding	Common	Rare
Deep dissecting haematomas	Characteristic	Rare
Haemarthrosis	Characteristic	Rare

as plasmin degrades the amino terminal of GP Ib–IX [191]. Thus platelet function is modulated by interactions with the components of the fibrinolytic system and in turn, platelets modify the fibrinolytic system.

Normal haemostasis is a balance between the procoagulant system and associated inhibitors and the fibrinolytic system and its inhibitors. A change in the delicate balance between clot formation and dissolution can tip the balance towards thrombus formation or haemorrhagic disorders.

3. Clinical presentation of bleeding disorders

Diagnosis usually requires careful examination of the patient history. Reviewing the medical history can establish if the disorder is hereditary or acquired. Abnormalities of platelet function are characterised by clinical bleeding and in most cases, mucocutaneous bleeding. The common presentation of cutaneous and mucosal bleeding lead to the term ‘purpuric disorders’. Haemorrhage into synovial joints as well as deep muscular haemorrhage is more common in severe hereditary coagulation disorders and rare in disorders of the vessels or platelets or in acquired coagulation disorders. The clinical findings useful in distinguishing between the two broad categories are given in Table 2 [192].

In acquired haemorrhagic disorders, the clinical picture is dominated by the underlying disorder. Multiple haemostatic defects are present in patients with acquired haemorrhagic diseases, which include thrombocytopenia as well as significant coagulation abnormalities. In most cases a history and physical examination will reveal the causes of thrombocytopenia, e.g. drugs or acute leukaemia. Similarly clinical examination is of value in the diagnosis of vascular disorders such as senile or allergic purpura and amyloidosis.

Von Willebrand disease, haemophilia A and B are the most frequent congenital bleeding disorders. The prevalence of haemophilia A and B in the general population of Western countries is approximately 1 in 20 000 and 1 in 100 000, respectively. Factor XI deficiency is the fourth most frequent, at least in countries with large Jewish communities. The remaining factor deficiencies that cause a bleeding disorder are much rarer and their prevalence is given in Table 3 [193].

4. Von Willebrand disease (vWD)

Professor von Willebrand published the first case of von Willebrand disease in 1926. The proband was a 5-year-old girl called Hjördis, who belonged to a family of 11 children. Both parents had troublesome nose bleeds. Three of the children died of bleeding from the gastrointestinal tract or mouth and another from haematemesis. The proband herself died from her fourth menstruation. Among the family members studied (a total of 35 females and 31 males), the predominant symptoms consisted of nose bleeding, bleeding from the gums and after tooth extraction, bleeding from female genital tract and bleeding from trivial wounds. Joint bleeding was relatively rare. The disorder has a greater penetrance in female patients due to increased haemostatic pressures of menstruation and pregnancy. Sadler’s classification of vWD is given in Table 4.

Table 4
Classification of vWD

Type I	Partial or quantitative deficiency of vWF
Type II	Qualitative deficiency of vWF
Type IIA	Selective absence of high molecular weight vWF multimers
Type IIB	Multimers with increased affinity for platelet GP Ib
Type IIM	Decreased platelet binding with normal high molecular weight vWF multimers
Type IIN	Decreased multimer affinity for FVIII
Type III	Complete deficiency of vWF Mixed phenotype caused by compound heterozygosity

Table 3
Prevalence of bleeding disorders

Deficiency	Estimated prevalence	Approximate number of reported cases	Gene on chromosome	Approximate number of reported mutations
Fibrinogen	1:1 million	300	4	<90
Prothrombin	1:2 million	40	11	<20
Factor V	1:1 million	150	1	<10
Combined factor V+VIII	1:1 million	40	18	<6
Factor VII	1:500 000	150	13	<80
Factor X	1:1 million	40	13	<25
Factor XIII	1:1 million	60	A subunit -6, B subunit -1	<20

By population based screening, approximate prevalence of vWD is 0.8–1.6 per hundred. Unlike the symptomatic patients registered at specialised treatment centres, relatively few patients identified by population screening have significant bleeding [194–197]. The prevalence of vWD as assessed by patients with bleeding symptoms varied from 0.02–0.11 cases per hundred [198,199]. In most studies the distribution of vWD types is 60–80% type I, 7–30% type II and 5–20% type III. The apparent distribution of type II vWD subtypes as shown by an analysis of 150 families in France was type IIA (30%), type IIB (30%), type IIN (28%) with the remaining 8% as either IIM or unclassified [200,201]. The highest prevalence of vWD type III, up to six per million, [202], has been documented in populations with a high incidence of consanguineous marriages.

vWD type I is the most common form of von Willebrand disease [203]. Type I vWD is characterised by a decreased level of structurally and functionally normal vWF and usually exhibits autosomal dominant inheritance. Subgroups of vWD type I are based on relative levels of plasma and platelet vWF [204]. vWD type I with exceptionally low vWF levels and normal multimer pattern, with impaired secretion of vWF has been reported. A candidate mutation, which replaces cysteine at position 386 with arginine resulting in the loss of cysteine pairing in the D3 domain, was identified. Family studies showed a dominant phenotype. The study also identified two additional unrelated patients, with a similar dominant type 1 phenotype, and another Cys367Phe mutation in the D3 domain [205]. The phenotype has been described in a further patient with a Cys367Tyr mutation [206]. To explain the dominant phenotype the authors proposed that the mutant subunit formed heterodimers with the normal vWF and was retained intracellularly and degraded [207]. Keeney et al. [208] investigated 12 unrelated patients with the dominant type 1 disease and found no mutations in the multimerisation domains. The latter authors also suggest that a modifier loci may have an effect on plasma vWF levels. Studies with a mouse model suggest that glycosylation defects may be associated with reduced half life but normal vWF multimer pattern [209]. It is likely that a similar mechanism can explain the effect of ABO and potentially other blood groups on plasma vWF levels [210]. Structures similar to ABO antigens have been demonstrated on vWF [211]. Though not directly studied in humans, altered clearance can potentially be an important factor in genetic variation of vWF levels [210]. Promoter polymorphisms may further contribute to vWF gene expression and quantitative reduction in vWF [212]. Casana et al. [213] reported a candidate mutation (Thr393Met) in seven affected individuals from the same family. Abuzenadah et al. [214] reported three propeptide (domains D1 and D2) missense mutations (Ser49Arg, Arg324Gln and Arg273Trp) associated with type I vWD in Turkish patients. Expression of recombinant vWF with the Arg273Trp mutation in COS cells showed that the secretion of the Arg273Trp mutation was severely impaired compared with the wild type recombinant vWF. However, the mutation did not affect the ability of vWF to form dimers in the endoplasmic reticulum though the formation of multimers was affected [215]. A review of other studies suggests that a proportion of symptomatic vWD type I is associated with compound heterozygosity for a recessive vWD type III null allele and another defect on the expressed allele [216]. Despite the high prevalence of type I vWD, the genetic cause remains elusive in most families. It is generally accepted that mutations in quantitative variants may lie across the whole vWF gene making detection a costly and painstaking process [217]. Segregation studies suggest that defects at loci other than the vWF gene may be the cause of the milder phenotype [218].

vWD type IIA includes patients with defective vWF dependent platelet adhesion caused by a shift in the vWF multimer distribution so that large multimers are absent and small multimers are abundant. Both dominant and recessive variants of vWD type IIA occur, though the dominant vWD type IIA is more common. The majority of mutations associated with type IIA vWD result in single amino acid substitutions, and the number continues to increase (vWF database—http://mmg2.im.med.umich.edu/VWF). Most of these point mutations lie within the posterior part of exon 28 in a 168 amino acid segment from 1505 to 1672 of pre–pro vWF (amino acid 742–909 of the mature subunit) and in the A2 domain of vWF protein. The A2 domain contains the Tyr842/Met843 proteolytic site [219]. Several of the type IIA mutations are close to this cleavage site or to the glycosylation site at Asn752 and may correspond to modified folding structure, which would affect its physiological function [220]. The most common mutation, Arg834Trp in the A2 domain, is thought to account for up to a third of type IIA vWD. Meyer et al. [221] identified two common mutations, Arg834Trp and Ile865Thr in 45 unrelated patients with type IIA phenotype. Several amino acid substitutions and other mutations have been identified in the anterior region of the exon 28 within the A1 domain in individual cases of type IIA vWD [221,222]. These regions have previously been shown to carry mutations associated with type IIB vWD.

Expression of mutant vWF sequences by transfection in mammalian cells has led to the classification of type IIA vWD into two distinct subgroups. In group 1 the point mutation leads to a defect in intracellular transport between the endoplasmic reticulum and the Golgi complex, with more profound effects on the secretion of high molecular weight forms [223]. Group 2 vWF released in tissue cultures is not different from wild type vWF in size distribution. The plasma of patients with type IIA vWD contains an increased amount of proteolytic fractions than in normal plasma. These observations led to the suggestion that the decrease of large multimers in group 2 patients is caused by excessive proteolysis [224]. Recently a vWF cleaving metalloproteinase that is responsible for depolymerisation of the multimers by limited proteolysis has been identified and characterised [225,226]. A study of ten patients from six families showed that the Arg552Cys mutation within the A1 domain induces

abnormal folding (possibly followed by intracellular retention) resulting in a type IIA like phenotype [227].

Several vWF defects result in impaired post-translational biosynthesis. vWD types IIC, IID, IIE, IIF, IIG and type IIH were reclassified as type IIA. These subclasses are characterised by the absence of large vWF multimers. Bands have mobilities different from normal plasma or some of the normal bands are absent. Type IIC is characterised by the loss of large vWF multimers and an increase in the quantity of vWF dimers. In one patient an insertion (625insGly) and a deletion leading to a premature stop codon (at position 711) and in a second, substitution Cys623Trp in domain D2 of the vWF propeptide (known to play a role in multimerisation) was identified [228]. Other candidate mutations Gly550Arg and Ins405AsnPro in the provWF sequence have been associated with the type IIC phenotype [229,230]. The variant previously classified as type IID is characterised by the absence of high molecular weight multimers and decreased proteolysis [231]. The underlying cause of the type IID subtype has been identified as a point mutation (Cys2010Arg) responsible for defective disulphide bonding at the C-terminal domains, thus impairing dimerisation [232]. Further mutations illustrating the importance of other carboxy-terminal cysteines (Cys2008Tyr, Cys2008Ser) in the normal dimerisation and their role in the assembly of multimeric vWF, resulting in a type IID phenotype, have been described [233,234]. Recently, Katsumi et al. [235] using a recombinant C-terminal domain of human vWF have suggested that Cys2008, Cys2010 and Cys2048 are the only cysteine residues that can form intersubunit bonds between vWF monomers in the cysteine knot like (a common motif that is found in the dimerisation region of several proteins) region of the vWF C-terminal. Dimerisation and intermolecular bonding of the vWF amino-terminal are processes that occur independently. A vWF molecule truncated after domain D3 still displayed intermolecular disulphide bonding [236]. This hypothesis supports the view that that the aberrant multimer pattern seen in type IID is the result of vWF multimerisation in the absence of normal vWF dimerisation. A single base deletion in exon 52 with Cys2010 as the first affected amino acid and impaired dimerisation was observed in a patient with type IIE phenotype [233].

Type IIB is associated with increased affinity of vWF for platelet receptor GP Ib. High molecular weight multimers (HMW) are usually absent from plasma but normal in the platelet pool and endothelial cells [237]. Platelet aggregation in the presence of ristocetin is enhanced. Mild thrombocytopenia is common in type IIB vWD and can be exacerbated during surgery, pregnancy or administration of arginine vasopressin. The increased affinity of the mutant form of vWF for platelets, is thought to cause spontaneous binding of large vWF to platelets in vivo, followed by clearance of the vWF and platelets. The remaining multimers are less haemostatically effective and bleeding occurs. Type IIB vWD is usually inherited as an autosomal dominant trait. Type IIB mutations are mostly confined to the anterior region of exon 28, corresponding to the vWF A1 domain, which contains the GP Ib binding site. Four mutations at loci Arg543Trp, Arg545Cys, Val553Met and Arg578Gln account for approximately 80% of the type IIB patients reported. Casana et al. [238] describe a 7-year-old girl, who had a first bleeding symptom at the age of 3 months, following her first vaccination. She was diagnosed with immune thrombocytopenic purpura as severe thrombocytopenia (9600–80 000 platelets/μl) was detected. She was treated with immunoglobulins and corticosteroids. The platelet count and haemorrhagic diathesis was not corrected and it was proposed that she undergo a splenectomy. The pre-operation tests revealed a prolongation of the activated partial thromboplastin time, decrease of vWF with low values in functional studies and absence of high molecular weight multimers. A high affinity for ristocetin dependent platelet function was confirmed and the patient was classified as having type IIB vWD. Sequencing revealed a 3941T → A transition, with Val551Asp substitution in exon 28 of the vWF gene [238]. An unusual type IIA phenotype with IIB genotype (Val551Phe) was described by Ribba et al. [239]. The patient plasma vWF showed the absence of HMW and intermediate multimers and decreased binding capacity to GP Ib though the phenotype was associated with intermittent thrombocytopenia. It has been suggested that this discrepancy between phenotype and genotype could be related to a loss of both high and intermediate multimers bound to platelets such that mutations may correspond to more severe forms of type IIB vWD. Different phenotypic expression of the same IIB mutation (Val553Met) has been reported within the same sibship. The propositus presented with a type IIB phenotype while his mother presented with a type IIA. Following administration of desmopressin a type IIB phenotype could be attributed to both [240].

Type IIM vWD variants are characterised by decreased platelet-dependent function in the presence of normal vWF multimer distribution, although multimer banding patterns on high resolution gels may be atypical. Mutations associated with type IIM vWD have been identified within the disulphide loop of the A1 domain, the region associated with platelet GP Ib binding [241]. Hillery et al. [242] described two family pedigrees with type IIM vWD. The index case in the first family presented in childhood with a lifelong history of increased bruising and moderately severe epistaxis; and she required arginine vasopressin therapy on several occasions. She also experienced bleeding 2 days post-tonsillectomy despite perioperative vWF replacement therapy. The other affected members of the family had an extensive history of increased bruising. The index case in the second family presented in infancy with a history of prolonged bleeding from the umbilical cord stump, and subsequently developed increased bruising and frequent severe epistaxis. The epistaxis required treatment with arginine vasopressin, vWF replacement therapy and/or cautery. The other affected family members have lifelong histories of increased bruising and moderately severe epistaxis during childhood. Multimeric analysis showed normal distribution

patterns. The affected members also showed a ristocetin cofactor induced agglutination:vWF Antigen ratio that was more than 2SD below the mean. The sequencing of genomic DNA identified two missense mutations in vWF exon 28. In the first family a mutation at nucleotide 4105T→A resulted in a Phe606Ile amino acid substitution. In the second family a missense mutation at nucleotide 4273A→T resulted in an Ile662Phe amino acid substitution. vWD type IIM "Vicenza" characterised by low vWF and the presence of supranormal multimers in plasma has been described in Italian and German families. Though recently two candidate mutations were identified, the first one in the D3 domain (Arg442His) and the other in the propeptide D2 domain (Met740Ile) the relationship between these mutations and the phenotype is unclear, though a reduced survival of vWF is thought to cause the reduction in plasma vWF [243–245].

Ribba et al. [246] identified two related patients (mother and daughter) with haemorrhagic syndrome, prolonged bleeding time, vWF antigen and vWF ristocetin cofactor activity at the lower limit of normal, normal levels of FVIII activity and normal multimer patterns. A Ser968Thr mutation was identified in vWF domain A3, and both patients showed decreased binding of vWF to collagen. A recombinant form of the vWF exhibited normal multimeric pattern and decreased binding to collagen.

The subtle differences observed in the vWD type IIA phenotypes are not reflected in the clinical presentation. The shared underlying defect of vWF multimerisation is common in different vWD types and subtypes. The multimeric pattern of vWF seen on high resolution sodium dodecyl sulfate-agarose electrophoresis shows that each multimer band consists of a triplet. Limited proteolysis at positions Tyr842–Met843 produces N- and C-terminal fragments which are contained within the vWF multimers and gives rise to the triplet structure [247]. Enayat et al. [234] describe a patient with a 8 bp deletion in exon 51 resulting in early termination of the polypeptide and loss of ten cysteine residues. The mutation leads to the synthesis of a smaller subunit and faster migrating multimer bands. Results also suggest that a common defect of vWF biosynthesis, lack of dimerisation, may cause diverse types and subtypes of vWD [232–234]. Theoretically, defects of vWF dimerisation can lead to the formation of multimers with an odd number of subunits along with the formation of multimers with even number of subunits, giving rise to different vWF multimer patterns.

Mutations causing types IIB, IIM and IIA vWD are located mostly in the A1 and A2 domains. Three dimensional structures provide a molecular interpretation of the mutations involved in type II vWD. Molecular modelling shows that most type IIB mutations are located near the predicted heparin site. Type IIM mutations correspond to spatially different set of amino acid residues. It is known that heparin binding inhibits the vWF–GP Ib interaction. One proposed molecular explanation of these mutations is that type IIB vWD involves decoupling of the inhibitory effects of heparin binding on the vWF–GP Ib interaction, leading to up regulation, whereas type IIM involves the disabling of GP Ib binding to vWF leading to down regulation [220,248]. In vitro studies show that shear induced platelet aggregation was increased in type IIB vWD and abolished in type IIM vWD [249]. Others hypothesise that vWD type IIB mutants bind to GP Ib receptors on platelets, blocking platelet adhesion to collagen, thus leading to bleeding symptoms [250].

vWD type IIN is an autosomal recessive disorder characterised by a marked decrease in the affinity of vWF for FVIII. Affected patients exhibit normal vWF multimers, vWF antigen, and ristocetin cofactor activity. Low plasma levels of FVIII are found as a result of reduced vWF binding and increased proteolysis of FVIII in circulation. Conventional coagulation screening tests may lead to the misleading diagnosis of haemophilia A. Although vWD type IIN is rarer than haemophilia A, the diagnosis should be considered in patients with mild or moderate FVIII deficiency if inheritance is not clearly X-linked. Type IIN vWD can be caused by at least 14 missense mutations localised within the N-terminus D′ and D3 repeats of the vWF, in a region shown to contain the FVIII binding domain. The Arg91Gln is the most common type IIN vWD mutation. The Arg91Gln, Arg53Trp, Thr28Met substitutions account for more than three quarters of all reported vWD type IIN cases. All of these mutations are localised in the first 102 amino acid residues of the mature subunit (764–865 of pre–pro-vWF) which correspond to the D′ domain. Expression of recombinant vWF carrying many of these mutations has confirmed their role in defective FVIII binding. These studies have shown that vWF carrying the Arg91Gln mutation retains some residual ability to bind FVIII, while Thr28Met and Arg53Trp mutations completely abolish binding [251–254]. Patients with type IIN mutations, which maintain residual FVIII binding, have higher FVIII levels in the circulation.

Jorieux et al. [255] report the case of a 74-year-old man referred because of a prolonged APTT and borderline bleeding time. The patient had experienced bleeding problems following tooth extraction and puncture of femoral artery, which was followed by the development of a haematoma. Following a diagnosis of vWD type II subsequent surgery (total hip replacement, tooth extraction, pacemaker implantation) was performed under treatment with vWF concentrates without any bleeding problems. Plasma FVIII activity and vWF antigen were below the normal range. His 66-year-old sister also had bleeding complications after hysterectomy and frequent ecchymosis. She had normal FVIII activity and slightly reduced levels of vWF antigen. Sequence analysis of the patient's vWF gene showed a heterozygous state with a G→A transition resulting in the substitution of Asn for Asp at position 116 of the mature vWF subunit and a C→T transition changing the codon Arg896 into a stop codon. His sister was found to be heterozygous for the Arg896 mutation only. Expression of the Asn116 mutation in COS cells confirmed that this mutation was responsible for the impaired FVIII binding and decrease of high molecular weight multimers. Studies with monoclonal antibodies directed against epitopes of

the D′ domain of the VWF suggested that conformational changes in the D′ domain were induced by the Asn116 mutation located in the D3 domain. The potential conformation change induced by the Asp116Asn substitution may also affect the formation of interchain disulphide bridges to decrease the concentration of vWF multimers. The nonsense mutation at Arg896 has been identified in the homozygous state in vWD type III patient and in a heterozygous state in a mild vWD type I phenotype [256]. The mixed phenotype of the patient highlights the complex nature of the multimerisation process, and that mutations lying outside the FVIII binding region, can lead to multimerisation defects and to loss in the ability to bind FVIII as a secondary consequence of conformational changes. In the study reported by Nesbitt et al. [257] reduced FVIII binding was relatively common in vWD type I, suggesting that the phenotypic presentation of vWD varied, and that mutations other than that found in the binding site for FVIII may influence FVIII binding ability. Mutations Cys25Arg, Cys25Tyr, Cys95Phe (in the D′ domain) and Cys462Arg (in the D3 domain) are further additions to atypical type IIN vWD associated mutations that cause defective FVIII binding and quantitative deficiencies and abnormal multimers [258]. Mutations in the D′ domain that reduce FVIII binding are thought to induce conformational changes in the D′ domain that also affect normal multimerisation and optimal secretion [259]. In the future, the use of genetic analysis and molecular modelling, based on vWF A1 domain crystal structures may assist diagnosis and provide further insights into the effect of particular mutations [260].

vWD type III is a recessive disorder in which there is no detectable vWF. The parents of affected individuals may be phenotypically normal or may present with a type I phenotype. A range of deletions, missense, nonsense and frameshift mutations predicted to result in loss of vWF protein expression or expression of a truncated protein have been identified in vWD type III families [29]. In a study of 37 multiethnic group of patients, 24 different gene alterations were identified. The majority of mutations caused null alleles, 11 being nonsense mutations, four small deletions, three possible splice site mutations, three candidate missense mutations, two small insertions and one large deletion [262]. Other mutations are found in the vWF database http://mmg2.im.med.umich.edu/vWF and are summarised in Fig. 3. Results indicate that molecular defects responsible for vWD type III are scattered throughout the entire vWF gene. A single cytosine deletion at position 2680 (exon 18) in a six cytosine stretch spanning nucleotides 2680–2686 of the vWF gene was reported in approximately 50% of 24 Swedish type III patients [261]. Other ‘hotspot’ mutations are found in arginine codons Arg896, Arg1090 and Arg1772 [262]. As in vWD type IIN, the absence of FVIII–vWF binding causes a secondary deficiency of factor VIII, so that spontaneous bleeding into joints and soft tissues may occur.

Platelet type vWD is caused by mutations of GP Ibα that increase its affinity for vWF. Platelet vWD is transmitted as an autosomal dominant trait. The original report describing this disorder studied five patients representing three generations of a single family. Bleeding times in the upper normal range in conjunction with low–normal platelet counts,

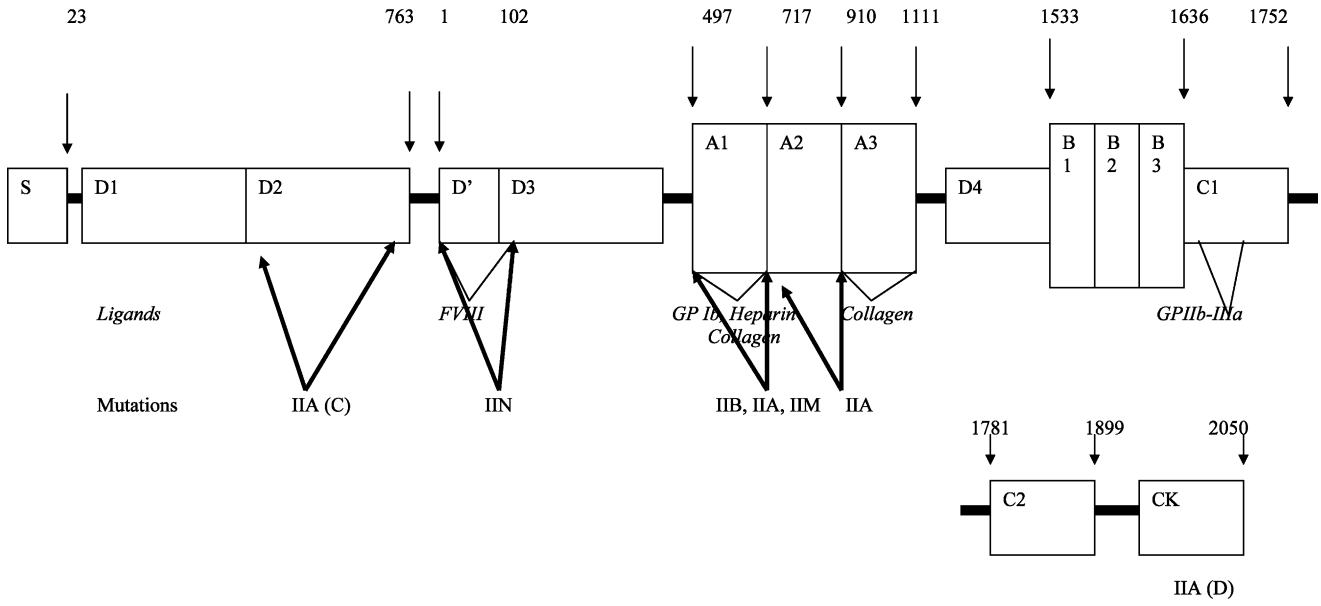

Fig. 3. The vWF gene located on chromosome 12, contains 52 exons and is transcribed into an 8.7 kb mRNA, which encodes 2813 amino acids pre-cursor, named pre–pro vWF, consisting of a 22 aa signal peptide, a 741 aa propeptide and a 2050 mature vwF subunit. The analysis of the amino acid sequence for a vWF precursor showed that over 90% of the sequence is contained within repeats of four types of homologous domains designated A to D and arranged as: D1 (coded for by exons 3–11), D2 (exons 11–17), D′ (exons 18–20), D3 (exons 20–28), A1, A2 (exons 28), A3 (exons 28–32), D4 (exons 35–39), B1–B3 (exons 40–42), C1 (exons 43–44), C2 (exons 45–48), C-terminal (exons 49–52).

normal FVIII coagulant activity, and selective decrease of HMW multimers was observed. Binding of patient vWF to washed normal platelets was within normal limits, whereas the binding of normal vWF to patient platelets was significantly increased. The bleeding diathesis was thought to result from rapid clearance from the plasma of HMW multimers of vWF. The platelet hyper responsiveness may be demonstrated with low concentrations of ristocetin. Whereas normal platelets show little or no aggregation at ristocetin concentrations of 0.5 mg/ml or lower, patient platelets show significant binding of vWF, together with strong aggregation [263]. Subsequently the mutation Gly233Val was described in one case [264], and Met239Val was described in another kindred. The latter mutation was also described in unrelated Japanese kindred and as occurring de novo in another patient [265–267]. Both mutations were close to each other in the primary structure of the polypeptide chain and within the Cys209–Cys248 disulphide loop. While it is possible that vWF may bind within this region, it is also conceivable that conformational changes within this region cause structural alterations thus facilitating the binding of vWF at more distant sites. A recombinant GP Ibα fragment containing the Met239Val mutation reproduced aspects of the platelet vWD phenotype. Spontaneous binding occurred between the mutant protein and vWF but not the WT GP Ibα and vWF. Low concentrations of ristocetin induced vWF binding to the immobilised mutant protein but not the WT GP Ibα [268]. Ligand binding studies and adhesion assays under flow conditions with recombinant GP Ibα proteins involving Gly233Val, Met239Val, or Val234Gly mutations, suggest that conformational changes within amino terminal of GP Ib influence the stability of the GP Ib–vWF interaction [269]. CHO cells expressing the mutant proteins—Gly233Val, Met239Val—were shown to roll more slowly on immobilised vWF than cells expressing the WT GP Ibα [270].

Acquired von Willebrand syndrome (AvWS) is a rare bleeding disorder with laboratory findings similar to those of congenital von Willebrand disease, but in patients devoid of both personal and family history of bleeding diathesis. In a survey of 186 patients, AvWS was associated with lymphoproliferative (48%), myeloproliferative (15%) disorders, neoplasia (5%), immunologic diseases (2%), cardiovascular diseases (21%) and miscellaneous conditions (9%). Bleeding time was prolonged, both APTT and FVIII were normal in some patients and vWF antigen was reduced but with a wide range of values [271]. In a single study of patients with haematologic disorders the frequency of type I vWD disease was 32.3% and type II was 67.7% [272]. Casonato et al. [273] reported a case of a patient with systemic lupus erythematosus who presented with severe bleeding complications following a kidney biopsy. vWF ristocetin cofactor activity and vWF antigen was low or undetectable with decreased FVIII coagulant activity. Multimer analysis detected the presence of two bands, one low molecular weight band and a high molecular weight band. An anti-vWF antibody was demonstrated in this patient and multimer pattern near-normalised following immunosuppressive therapy. The functional assays measured as ristocetin cofactor activity or as collagen binding activity, appear to be the most sensitive method of measurement of vWF defect in AvWS. Plasma multimers were missing in a majority of analysed cases. Three main mechanisms have been demonstrated to account for AvWS: circulating autoantibodies to vWF, adsorption of vWF onto tumoral or activated cells and proteolytic degradation of vWF [274,275].

5. Bernard–Soulier syndrome

Abnormalities of the GP Ib–IX–V complex are associated with abnormal platelet function and appearance, giving rise to a syndrome first described by Bernard and Soulier in 1948. BSS is an autosomal recessive disorder characterised by moderate to severe thrombocytopenia, enlarged (giant) platelets, and a tendency to have perfuse and often spontaneous bleeding. BSS platelets aggregate normally in response to ADP and collagen but do not aggregate when platelet rich plasma is stirred with ristocetin or botrocetin. Mutations of the genes for GP Ibα, GP Ibβ, and GP IX have been identified in patients with BSS. Most of these mutations are in the GP Ibα region and decrease the expression of GP Ibα on the platelet surface, although mutations that affect function more than expression of GP Ibα have also been identified. Mutations of GP IX and GP Ib generally decrease expression of the entire complex on the platelet surface. A summary of mutations causing BSS is found in http://www.bernard-soulier.org/mutations (Fig. 4).

Kenny et al. [276] describe two siblings, who presented at 15–16 months of age with easy bruising and episodes of bleeding. Platelet aggregation studies showed normal response to ADP, epinephrine, collagen and arachidonic acid with no response to ristocetin. Bleeding time was prolonged, >13 min. Their platelet count varied from 75 000 to 120 000 mm^3. Large platelets were observed on a blood smear. DNA sequence analysis showed that the affected individuals were compound heterozygotes for two mutations. One inherited from a maternal allele, a T777→C point mutation in GP Ibα converting Cys65→Arg within the second leucine rich repeat. The other, a single nucleotide substitution G2078→A for the tryptophan codon TGG causing a nonsense codon TGA at residue 498 (also described by Holmberg et al. [277]), within the transmembrane region of GP Ibα, inherited from a mutant paternal allele. Although GP Ibα was not detected on the surface of the patient's platelets, soluble GP Ibα could be immunoprecipitated from the plasma. GP IX was normal and GP Ibβ was present in reduced amounts. The expression of the Cys65Arg GP Ibα mutant in CHO cells stably expressing the GP Ibβ–IX complex was similar to the WT GP Ibα, though the mutant protein did not bind vWF. When plasmids containing Trp498stop GP Ibα mutation were transfected into the same CHO cells, surface expression of GP Ibα was not observed. Thus, the compound

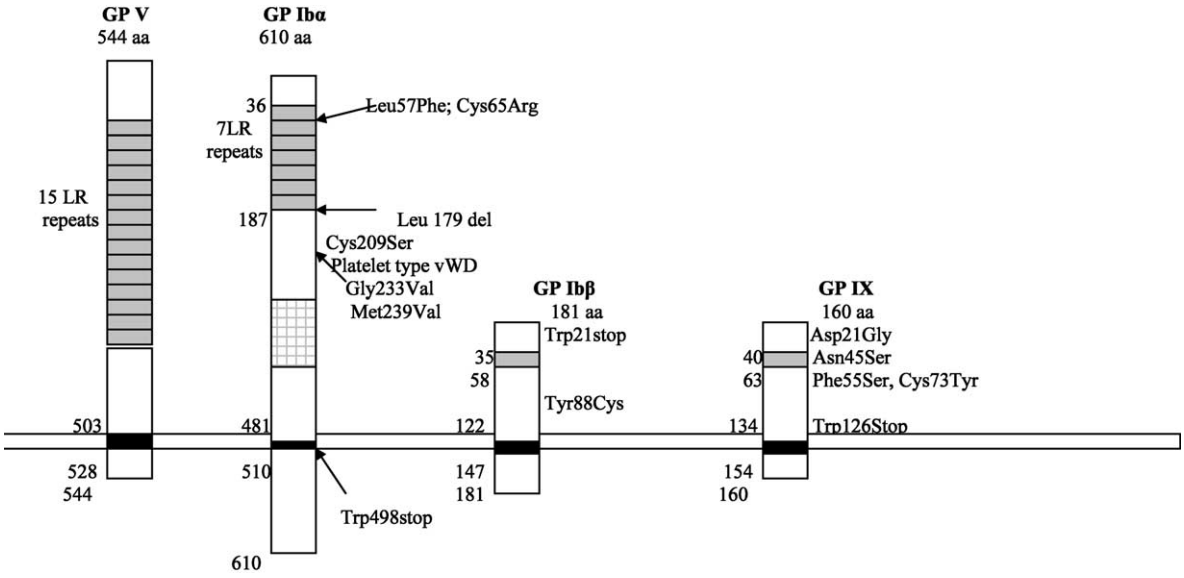

Fig. 4. Schematic representation of the GP Ib–IX–V complex. The amino terminus of the proteins are oriented towards the outside and the carboxyl terminus are oriented towards the inside of the cell. The hatched area indicates carbohydrate rich regions. Numbering of the amino acids for the mature proteins is indicated. The leucine rich (LR) repeat sequences, and amino acid residues, are indicated as shaded rectangles. Amino acid substitutions that cause BSS and platelet type vWD are shown. The N-terminal region of GP Ibα is implicated in the binding of vWF. The N-terminal disulphide loop and adjacent leucine rich repeats (His1–Ala200), the C-terminal disulphide loop region (Phe201–Gly268), and an anionic, tyrosine sulphated sequence Asp269–Glu282 are the threes sites thought to be important for the interaction with vWF. Two disulphide loops are formed between Cys209–Cys248 and Cys211–Cys264.

heterozygous defect produces Bernard–Soulier syndrome by a combination of synthesis of a non-functional protein and truncated protein that fails to insert into the plasma membrane and is found circulating in the plasma. Molecular characterisation of two further patients with BSS, detected a deletion of the last two bases of the codon for Tyr492 which shifted the translation reading frame to produce a GP Ibα polypeptide with novel transmembrane domains. The mutations affected the anchoring of the polypeptide in platelets [278,279]. A point mutation found in codon 129 of GP Ibα resulted in the substitution of Pro for Leu in two related individuals with well established BSS. Patient platelets exhibited about 40% of normal vWF binding and 40% of normal GP Ib–IX surface antigens. The described mutation affects the conformation of the GP Ib–V–IX receptor and alters its availability on platelet surfaces [280]. Molecular genetic analysis performed on other patients with BSS revealed the following changes: Leu57Phe [281]; Ala156Val [282], Leu179del in the leucine rich repeat sequences [283]; Cys209Ser [284], Trp343stop [285] and Ser444stop [286]. Mutations in leucine rich motif of GP Ibα appear to cause BSS by disrupting the structure of the α subunit and hence the binding of vWF. Other mutations are due to premature termination, which result in a truncated protein or frameshifts, which result in the protein not being inserted in the platelet membrane.

In another patient diagnosed with BSS, GP Ibβ gene was deleted from one chromosome. There was a single nucleotide deletion within the codon for Ala80 in GP Ibβ in the other allele. GP Ibα, GP Ibβ and GP IX were not detected on the patient's platelets. When WT GP Ibβ was transfected into CHO cells there was a marked increase in the surface expression GP IX, when compared with cells transfected with mutant GP Ibβ. The authors suggest that the absence of detectable GP Ibα on the patient's platelets is secondary to the defective interaction of the mutant GP Ibβ subunit with GP IX [287]. Other reports of BSS include a single base G→A substitution that causes a Trp21stop change with premature termination of translation of the GP Ibβ protein [288]. A further patient was a compound heterozygote for two single nucleotide substitutions leading to Tyr88Cys and Ala108Pro mutations in the GP Ibβ protein. The patient was described as having a variant form of BSS in which giant platelets were present but neither thrombocytopenia or a definite tendency to bleed was observed. There was a reduced density of GP Ib–IX complexes on the platelet surface [289]. One hypothesis is that the reported mutations in GP Ibβ cannot sustain the disulphide bridge between GP Ibα and GP Ibβ. Ludlow et al. [290] reported a patient with mutation within the promoter region of GP Ibβ, which was unmasked by a deletion on chromosome 22 in the other allele, resulting in a DiGeorge/velo-cardiofacial syndrome with BSS.

Mutations of GP IX have been described which result in the Bernard–Soulier phenotype. Clemetson et al. [291], describe a patient with bleeding symptoms together with absence of platelet agglutination to ristocetin, whereas aggregation to ADP, collagen and arachidonic acid was normal. Surface labelling of platelets detected reduced amounts of GP IX, GP Ibα, GP Ibβ and GP V. Sequence analysis after polymerase chain reaction detected an A→G

conversion, with Asn45Ser conversion in both GP IX alleles. Molecular defects were determined in other cases of BSS with decreased expression of GP IX as determined by immunofluorescence or flow cytometric analysis. In four cases, Cys73Tyr and in a single case Phe55Ser substitution was identified [292,293]. In a further case of BSS, GP IX was completely absent but residual amounts of GP Ibα and GP V were detectable. A point mutation with a Trp126stop conversion was identified in this patient [294]. GP IX mutations described in three siblings with BSS, Asp21Gly, and Asn45Ser were introduced by site directed mutagenesis into cDNA for GP IX. Unlike WT GP IX, neither of the mutants was able to increase GP Ib expression on the cell surface when co-transfected with GP Ibα and GP Ibβ into CHO cells [295,296]. Lanza et al. [297] describe a Leu7Pro mutation in the signal peptide of GP IX which abolished the surface expression of the GP Ib–V–IX complex. Most mutations are within or flank the single leucine rich residue of GP IX. These findings suggest the importance of the leucine rich motif on GP IX in the stability of the GP–Ib–IX complex.

There are no reports of BSS affecting the GP V gene. Furthermore, mice without GP V had no BSS like symptoms [55].

6. Glanzmann thrombasthenia

Glanzmann reported a case of a bleeding disorder, starting immediately after birth, characterised by prolonged bleeding time and abnormal clot retraction. He named the disease 'thrombasthenia'. Glanzmann's thrombasthenia (GT) is an autosomal recessive disorder. The hallmark of this disease is severely reduced or absent platelet aggregation in response to multiple physiological agonists, such as ADP, thrombin or collagen. The disease is caused by mutations in the genes encoding GP IIb or GP IIIa that result in qualitative or quantitative abnormalities of the proteins. GT patients are grouped into three types according to the amount of GP IIb–IIIa complex expressed on their platelet surface: less than 5% of GP IIb–IIIa for type I patients, 10–20% for type II patients and 50–100% for type III (variant) patients. In recent years, the number of mutations that have been identified have increased and are found in the internet database; http://med.mssm.edu/glanzmanndb and are summarised in Fig. 5 [298]. Patients with GT present with a wide spectrum of symptoms varying from mild to severe bleedings. The large number of mutations found in the database offers an opportunity to investigate phenotype/genotype correlations [299].

Studies indicate that the minimal ligand binding structures are located in the amino terminal half of GP IIb–IIIa. A recombinant GP IIb–IIIa fragment that was composed of residues 1–233 of GP IIb and residues 111–318 of GP IIIa has been shown to bind to an RGD containing peptide [300,301]. The identification of regions within each subunit that bind ligands involved the use of ligand mimetic peptides

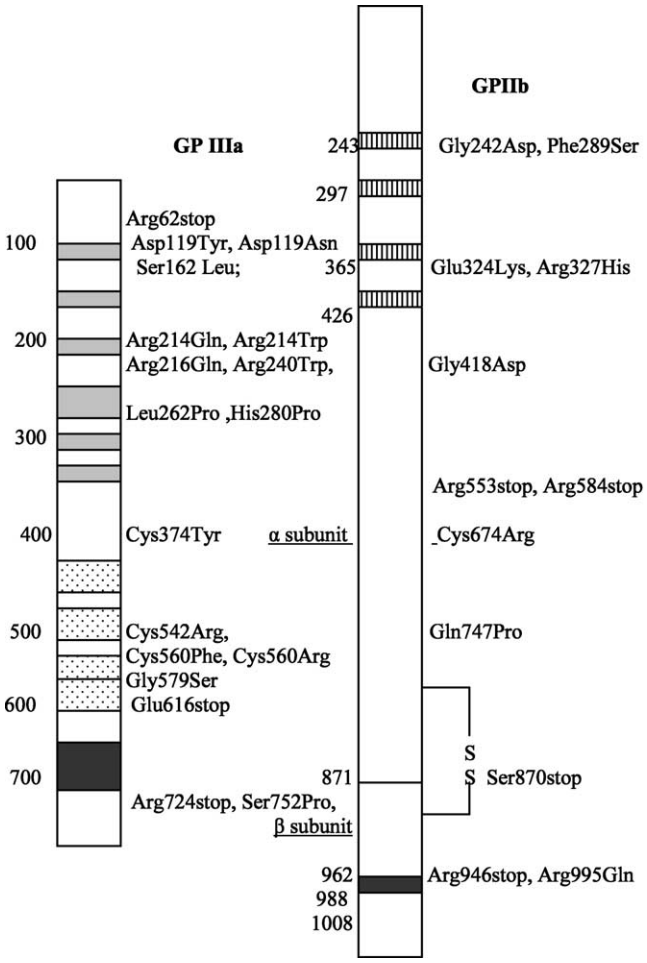

Fig. 5. Schematic map of GP IIb–IIIa. The larger and smaller subunits of GP IIb have been termed α and β. The filled area represents a possible transmembrane domain. The shaded areas represent homologous segments. The spotted areas represent cysteine rich repeats. Areas with vertical lines indicate Ca^{2+} binding sites. Missense mutations are shown.

as well as monoclonal antibodies specific for a given region, which inhibit ligand binding. Two distinct, discontinuous regions within GP IIIa have been implicated in the ligand binding function of the receptor. RGD (Arg–Gly–Asp) peptides cross link to the region defined by Asp109–Glu171. A mutation Asp119Tyr results in complete loss of ligand binding function, as do mutations in the near vicinity [302,303]. A potential fibrinogen interactive site in GP IIIa is defined by residues Ser211–Gly222. Natural mutations at Arg214 inhibit fibrinogen binding [304,305]. The importance of these regions, Asp119, Ser121, Ser123 (DXSXS motif), and Asp217 is likely because of their participation in the formation of a MIDAS. Glu220 is a further residue identified by mutagenesis as essential for the ligand binding function of GP IIIa [306]. Peptides derived from the carboxyl terminal of the fibrinogen γ chain cross link to the GP IIb region Ala294–Met314, the area that includes the second cation binding region of GP IIb [307,308]. The studies demonstrate that for GP IIb–IIIa, integrin-ligand recognition

requires the cooperation between IIb and IIIa subunits. The two peptides (RGD sequence, and the γ chain sequence) may bind to separate but allosterically related sites [309]. The structural model of the ligand-binding domain of the GP IIb chain has been predicted by computer modelling. The ligand binding sequence of GP IIb is composed of the amino-terminal 450 amino acids with seven homologous repeats which contain four cation binding sites. A recent computer model proposes that the seven N-terminal repeats adopt the fold of a β-propeller domain. The domains contain seven, four stranded β-sheets or blades arranged in a torus around a pseudosymmetry axis [61,310]. The method by which ligands interact with GP IIb–IIIa is not known, though site directed mutagenesis implicated residues at the top of the β-propeller model in ligand binding [311]. Nevertheless the model appears to contradict the data implicating the direct involvement GP IIb cation binding motifs in ligand binding, by placing them spatially distant from ligand contact points on the upper surface of the propeller [312].

Ambo et al. [313] describe three patients from different families, with normal platelet counts, and prolonged bleeding times (>15 min). Platelet aggregation to ADP, collagen and epinephrine was impaired, but platelet response to ristocetin was normal. Clot retraction was within the normal range. The level of GP IIb–IIIa, as measured by flow cytometry varied from 7 to 20%. The patients were diagnosed with type II GT. Three single nucleotide substitutions resulting in His280Pro, Cys560Phe and Gly579Ser missense mutation were identified in GP IIIa. One patient was homozygous for His280Pro mutation and the other two compound heterozygotes for this mutation. Ectopic expression of wild type and mutant complexes in CHO cells confirmed decreased surface expression of the mutated GP IIb–IIIa complexes. Molecular analysis of three family members with GT type I revealed a 6 bp deletion with amino acid deletion/substitution (Ile325Pro326Gly327→Met) in GP IIIa which prevented heterodimerisation and surface expression of the GP IIb–IIIa receptor complex [314]. A mutation at Leu117Trp in the GP IIIa chain resulted in the intracellular retention of the malfolded GP IIa–IIIb heterodimers [315]. Bajt et al. [316] report a patient diagnosed with GT whose platelets contained near normal amounts of GP IIb–IIIa, which failed to bind fibrinogen. DNA analysis revealed the amino acid substitution Arg214Gln in GP IIIa. In a further GT patient, a point mutation in GP IIIa resulted in Ser162Leu substitution with delayed trafficking of the unstable mutant complex [317].

Ruiz et al. [318], describe a patient with a clinical history of very mild bleeding, with rare gum bleeding but neither epistaxis nor bruising, with a surface expression of GP IIb–IIIa about 20% of normal. The diagnosis of GT was based on severely reduced platelet response to a series of agonists. Sequence analysis detected a homozygous Cys560Arg mutation in GP IIIa, leading to a gain of function mutation, which was able to spontaneously bind fibrinogen. Whether the mutation affects receptor trafficking is not known. Other evidence suggests that the cysteine rich domain of GP IIIa might be involved in GP IIb–IIIa activation. Reduction of disulphide bonds by dithiothreitol can activate GP IIb–IIIa [319]. Disruption of the long-range Cys_5–Cys_{435} disulphide bond of the IIIa subunit with the Cys5Ala and Cys435Ala mutations resulted in a GP IIb–IIIa complex capable of constitutively binding soluble fibrinogen [320]. The results show that ligand binding can be influenced by long range constraints within GP IIb–IIIa. In addition a misfolded extracellular domain may have secondary effects on bi-directional signalling. Patients with GT phenotype and normal expression of GP IIb–IIIa with defective inside–out signalling have been identified [321].

Investigation of a further patient with a type II thrombasthenia phenotype with a platelet GP IIb–IIIa value about 10% of the normal detected compound heterozygosity for the GP IIb gene with an exon5–intron 5 splice site mutation inherited from the father and a Cys674Arg inherited from the mother. Virtually all of the proband's GP IIb-mRNA was accounted by the allele inherited from the mother showing that the mRNA from the splice site mutation was unstable. Transfection experiments showed that surface expression of the GP IIb–IIIa complex was strongly attenuated in cells expressing normal GP IIIa with mutant GP IIb. Thus the intramolecular 674–687 disulphide bridge appears to be required for the normal processing of the GP IIb–IIIa complexes [322]. A two amino-acid insertion (Arg–Thr between residues 160–161 of the GP IIb subunit), within the Cys146–Cys167 loop leads to variant GT, with normal expression of platelet GP IIb–IIIa, which did not bind fibrinogen. The mutation is found on the upper surface of Springer's β-propeller model. The patient had been suffering from easy bruising since birth, and severe nasal bleeding at irregular intervals. Red cell transfusion had been required several times. Menorrhagia had been noticed since her first menstruation. Prolonged bleeding time and normal platelet count was observed. Platelet aggregation was absent in response to ADP, epinephrine, collagen and thrombin, however, ristocetin induced platelet aggregation was normal [323].

A Leu214Pro mutation in GP IIb was identified in a 55-year-old man who suffered from repeated bouts of epistaxis, excessive bleeding after dental extractions and severe episodes of pharyngeal and gastric bleeding. Surface expression of the mutant receptor in CHO cells was ≈60% of normal, but these receptors did not adhere to immobilised fibrinogen, suggesting qualitative and quantitative abnormalities [324]. Other naturally occurring mutations that impair surface expression and abrogate ligand binding are Pro145Ala and Pro145Leu mutation in GP IIb [325].

Functional analysis of mutation Glu324Lys in GP IIb, showed that the mutation prevented heterodimerisation of GP IIb and IIIa and was responsible for the GT type I phenotype [326]. Sequence analysis of a platelet GP IIb

polymerase chain reaction amplified mRNA, isolated from a GT patient, revealed a Gly418Asp amino acid substitution near the fourth calcium binding domain of GP IIb. Studies indicated that that the biosynthetic defect occurred in the pre-Golgi compartment [327]. Since then it has been demonstrated that residues flanking the calcium binding domains are needed for proper folding of the GP IIb–IIIa, prior to transport to the Golgi apparatus. The Gly273Asp mutation found in the first GP IIb calcium binding domain similarly impaired transport from the endoplasmic reticulum to the Golgi complex [328].

Two mutations within the cytoplasmic domain of GP IIIa, resulting in GT have been identified. The mutations are Arg724stop and Ser752Pro. Resting platelets from both patients showed significant levels of GP IIb–IIIa that did not bind to fibrinogen after platelet activation by agonists. Binding of fibrinogen was triggered by modulators (Arg–Gly–Asp–Ser peptide) of GP IIb–IIIa that induce an active conformation of the complex. Functional analysis of CHO cells stably expressing the Arg724stop mutant revealed that the complex is able to mediate binding to immobilised fibrinogen, though downstream events such as cell spreading did not occur. The authors suggest that the observations indicated the importance of the cytoplasmic domain in bi-directional transmembrane signalling in platelets [329,330]. Baker et al. [331] developed a method to create GT variants in CHO cells expressing a constitutively activated form of GP IIb–IIIa. Using this approach they identified, Glu312Lys, Gly331Glu, Ser334Phe mutations in GP IIIa, in the activation defective variants. These mutations do not inhibit ligand binding (binding of fibrinogen mimetics in the presence of activating antibodies) or cell spreading when plated on fibrinogen, but appear to interfere with inside-out signalling.

Acquired thrombasthenia, with haemorrhagic diathesis, and characteristic platelet aggregation studies can be induced by autoantibodies directed against a normal GP IIb–IIIa receptor. Typically this disorder develops during adulthood, and has been associated with lymphoid neoplasms [332].

Molecular characterisation of GT indicates the diversity of mutational defects that cause GT. Point mutations leading to single amino acid substitutions, insertions or deletions, nonsense mutations or splicing abnormalities have been reported in both genes coding for GP IIb–IIIa. The mutations lead to defects in mRNA splicing, mRNA stability, subunit association and intracellular trafficking, and either directly or indirectly (through conformational changes) affect ligand binding or signal transduction.

7. Other collagen receptor defects

Moroi et al. [333,334] reported two cases of GP VI deficiency one of which was a 56-year-old Japanese woman with recurrent purpura and epistaxis since childhood. She developed excessive post-operative bleeding following hysterectomy. Platelet counts and clot retraction was normal, though she showed slightly increased bleeding time. Shape change, aggregation and ATP release was absent in response to collagen. Adhesion to coated collagen was mildly affected, in spite of only 10% expression of GP VI. Expression of GP Ia–IIa was normal. The second case was a 26-year-old woman with a tendency to develop purpura, occasional hypermenorrhagia and severe nose bleeds. The patient's platelets contained ≈50% of the normal GP VI. Although the human gene sequence for GP VI is now known, the molecular basis for the absence of GP VI in these patients is not known.

Nieuwenhuis et al. [335,336] described a patient with prolonged bleeding times, chronic mucocutaneous bleeding, defective in vitro platelet adhesion to collagen and absent in vitro collagen induced aggregation. The patient also had selective deficiency of the GP Ia subunit resulting in the absence of platelet surface GP Ia–IIa integrin. Platelets from a further patient with severe bleeding disorder also lacked GP Ia. Platelet aggregation in response to ADP, arachidonic acid, thrombin, epinephrine and ristocetin was normal. In contrast aggregation in response to collagen was defective. The response to collagen recovered after menopause [337,338]. In women, platelet adhesion to type I collagen showed a biphasic periodicity in synchrony with the menstrual cycle. Human megakaryocytes also express the oestrogen receptor. These observation support the hypothesis that sex hormones affect platelet function [339]. Subsequently patients were described with bleeding disorders arising from the development of inhibitory antibodies against GP Ia–IIa [340] or acquired deficiency of the integrin associated with myeloproliferative disorders [341].

Although, the levels of GP Ib–IX and GP IIb–IIIa on platelets can vary from one individual to the next, these differences never exceed a fraction of the mean population level [342]. On the other hand the number of GP Ia–IIa molecules varies widely (up to 10-fold) and correlates with quantitative estimates of platelet adhesion to collagen [343]. These differences correlate with certain allelic combinations of the GP Ia gene [344] and may be associated with dimorphisms within the 5′ regulatory region, which lead to diminished expression of the GP Ia gene [345]. It has been shown that platelet adhesion to collagen surface under flow depends on the density of GP Ia–IIa on the platelet surface as well as plasma and platelet levels of vWF [346]. The varying levels of GP Ia–IIa may have little significance in individuals who are otherwise haemostatically normal but may become more important in individuals who are otherwise predisposed towards bleeding by a genetic condition such as von Willebrand disease. In patients with vWD type I, at any given level of ristocetin cofactor activity the time to closure in a model system (platelet function analyser) varied inversely with collagen receptor density. This may account for the variability between patients with

similar levels of vWF antigen but different bleeding histories [347].

8. ADP receptor defects

Cattaneo et al. [348] described a 49-year-old man with a lifelong history of bleeding, prolonged bleeding time and severely impaired aggregation response to ADP. Platelet aggregation was reversible in response to weak agonists and impaired in response to collagen or thrombin. Other observations reported in these patients were (i) normal shape change and normal to mildly reduced mobilisation of cytoplasmic ionised calcium; (ii) no inhibition by ADP of PGE1 stimulated platelet adenyl cyclase but normal inhibition by epinephrine; (iii) reduced binding of the ADP analogue 2-methylthioadenosine 5′-diphosphate (MeS-ADP). The many similarities between this patient and a further second unrelated patient [349] suggest that they have a similar abnormality of ADP receptors associated with the receptor $P2Y_{12}$. Analysis of the second patient detected a two nucleotide deletion at amino acid 240 which led to a 28 amino acid residue shift and a premature stop codon. The second allele was silent, though the mechanism by which the wild type allele was repressed was not clear [142]. $P2Y_1$ is normally present in the platelets of these patients and is responsible for the residual response to ADP [350]. Cattaneo et al. [351] studied a family with two sisters with a defect in the $P2Y_{12}$ receptor. Both patients presented with a lifelong history of easy bruising, menorrhagia and severe bleeding complications after dental extractions or major surgery. Platelet function studies were similar to the two previous patients.

The son of one of these patients who was an obligate heterozygote had a number of binding sites for [^{32}P]2 MeS-ADP, which was intermediate between normal and that of the two patients. ADP induced a normal primary wave of aggregation and partially inhibited adenylate cyclase. A variety of agonists including collagen induced impaired or borderline low secretion of ATP from his platelets. This secretion defect was not caused by low concentrations of platelet granule contents. The authors argue that the patient presentation is similar to the ‘primary secretion defect’ and that the some of the patients with primary secretion defect may in fact be heterozygotes for a defect in $P2Y_{12}$. The bleeding history is mild in patients with primary secretion defect and was negative in this patient, probably because due to his youth he had never been exposed to situations at risk for bleeding [351]. Recently, two preliminary reports of congenital defects of platelet $P2Y_1$ and $P2X_1$ receptors, both associated with abnormal platelet aggregation and bleeding diathesis were presented [352,353]. The propositus of the family with the $P2X_1$ mutation was a 6-year-old girl with pronounced nose bleeds, which resulted in low Hb levels, low plasma iron and increased reticulocyte numbers. The mutation was identified as the loss of one leucine residue in the second transmembrane domain of the receptor [354].

9. Other receptor defects

Hirata et al. reported a Japanese patient with Arg60Leu mutation in the first cytoplasmic loop of the TXA_2 receptor in a mild bleeding disorder characterised by defective platelet aggregation responses to TXA_2 and its analogues. In cultured cells the Arg60Leu mutant of TPα was shown to impair PLC activation and adenyl cyclase activation [355]. Patients who are both homozygous and heterozygous for this mutation have been described. All patients had a lifelong history of mucosal bleeding and easy bruising but no episodes of major bleeding including haematuria, gastrointestinal bleeding and haemarthrosis. The platelets of the patients showed abnormal aggregation to ADP, collagen, arachidonic acid and thromboxane analogues. The heterozygous patients did not lack PLC activation, while the homozygous patients did [356].

The widespread tissue distribution of PAR1 and PAR4 suggest a multifactorial role for both receptors. Perhaps because of this and its role in foetal development, inherited defects of these receptors have not been described [357].

10. Signal transduction defects

The guanine nucleotide binding regulatory proteins (G proteins) play a major role in signal transduction from surface receptors and regulate downstream responses such as aggregation and secretion. Human platelets contain at least ten forms of the Gα including members of the G_i family ($G_{i1\alpha}$, $G_{i2\alpha}$, $G_{i3\alpha}$ and $G_{z\alpha}$) and the G_q family ($G_{q\alpha}$, $G_{16\alpha}$) and the G_{12} family ($G_{12\alpha}$ and $G_{13\alpha}$) as well as $G_{s\alpha}$ [146]. Various G protein-coupled receptors are illustrated in Fig. 6. It is the selectivity of the G proteins that determines which pathways are activated by a particular agonist. Brass [146] observes that the relative abilities of receptors to cause platelet activation seem to be related to their efficiency of coupling to different G proteins. Strong agonist potency seems to correlate best with ability to activate PLC. Weaker agonists such as ADP and epinephrine depend on their ability to inhibit cAMP formation. Pharmacological studies with selective receptor agonists and antagonists, combined with selected knockouts of receptors and G proteins have provided insights into early events in platelet activation. However, many features of the signalling process still remain a puzzle. The links from the G protein coupled pathways to receptor activation, aggregation and secretion are still poorly understood. Work with inhibitors suggests that low molecular GTP-binding binding proteins play a central role. Inhibitors of protein kinase C, RhoA, PI 3-kinase and Syk as well as general inhibitors

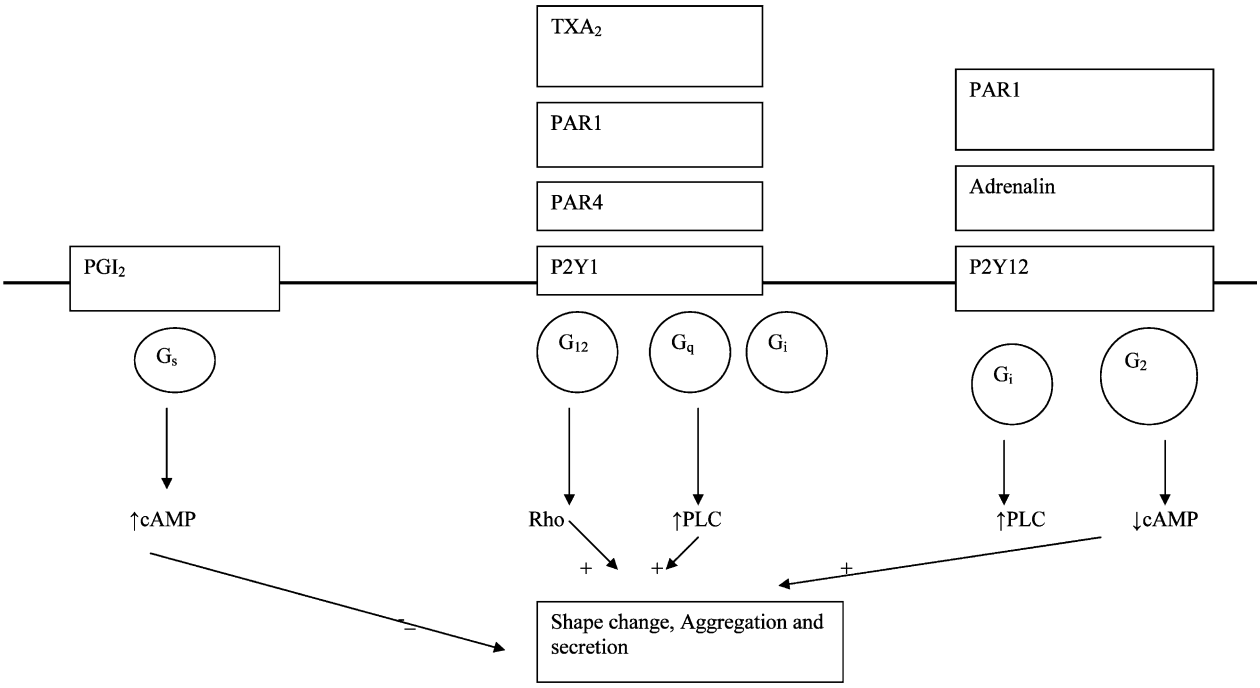

Fig. 6. Schematic representation of signalling pathways of G protein dependent agonists. The diagram shows early events in platelet activation. Several examples of heterotrimeric G proteins and G protein coupled receptors are shown. Classically G-protein receptors activate phospholipase C. This leads to the hydrolysis phosphotidylinositol 4,5-biphosphate to yield 1,4,5 inositol triphosphate and diacylglycerol. This raises cytosolic calcium by releasing sequestered calcium from the dense tubular system and also activates protein kinase C. Further, downstream signalling events that couple G protein activation to phosphorylation of pleckstrin, and other tyrosine kinases (e.g. Syk and Src related kinases) have not been identified.

of protein tyrosine kinases inhibit platelet aggregation [358].

Evidence is beginning to emerge that in some patients with platelet aggregation defects, the abnormalities lie in early signal transduction events. Several patients have been identified with relatively mild bleeding problems, and impaired dense granule secretion although their platelets have normal granule stores and generally synthesise substantial amounts of TXA_2. The bleeding times were prolonged on most occasions. Aggregation response to several agonists including ADP, epinephrine, collagen, thromboxane analogues, platelet activating factor were diminished and generally characterised by the absence of the second wave of aggregation. Exposure of patient platelets to cell permeable agonists (protein kinase C activators and ionophores), which presumably bypass early signalling events, induces normal secretion, suggesting that the process of exocytosis is essentially intact in these patients [359,360].

Gabbeta et al. [361] describe a 46-year-old female with mild life-long mucocutaneous bleeding diathesis associated with prolonged bleeding times and normal platelet counts. Studies on the patient showed the following: (i) platelet aggregation and secretion was abnormal in response to ADP, epinephrine, collagen and TXA_2; (ii) ADP and ATP contents of dense granules were normal; (iii) TXA_2 production in response to ADP and thrombin was diminished but was normal on exposure to arachidonic acid; (iv) calcium mobilisation was abnormal in response to thrombin and ADP, though calcium release induced by exogenous inositol triphosphate was normal. Immunoblot analysis of Gα subunits in the patient's platelet membranes showed a decrease in $G_{q\alpha}$ but not $G_{i\alpha}$, $G_{z\alpha}$, $G_{12\alpha}$, and $G_{13\alpha}$. Similar platelet abnormalities have been reported in $G_{q\alpha}$-deficient knockout mouse [148].

Platelets from one patient with a mild inherited bleeding disorder and abnormal platelet aggregation and secretion, showed abnormal signal transduction events such as reduced generation of IP3, mobilisation of intracellular calcium and phosphorylation of pleckstrin in response to several G protein mediated agonists. The amount of PLC isoforms in normal platelets decreased in the order PLC-γ2 > PLC-β2 > PLC-β1 > PLC-γ1 > PLC-δ1 > PLC-β4. Activation of PLC-β isoforms is dependent on $G_{q\alpha}$ or βγ subunits of heterotrimeric G proteins. The patient showed a selective decrease in PLC-β2 isoenzyme [362]. The PLC-β2 mRNA was decreased in patient platelets but not in neutrophils, though the PLC-β2 cDNA sequence showed no abnormalities, suggesting a lineage specific defect in PLC-β2 gene expression [363].

A 16-year-old male with lifelong mucocutaneous bleeding manifestation and abnormal platelet aggregation and secretion to multiple agonists was described by Gabbeta et al. [364]. Pleckstrin phosphorylation by protein kinase C is thought to be one of the events regulating GP IIb–IIIa activation. Pleckstrin phosphorylation was diminished in response to platelet activating factor and thrombin. The number of resting GP IIb–IIIa was normal though the

number of activated GP IIb–IIIa in response to activation by platelet activating factor, ADP and thrombin receptor agonist peptide (SFLLRN) was reduced. The authors suggest that the patient's platelets have a defect in inside–out signal transduction dependent GP IIb–IIIa activation.

Two unrelated families with a novel functional polymorphism leading to the over expression of the paternally imprinted extra large $G_{s\alpha}$ gene have been described [365]. The patients clinical symptoms are enhanced trauma related bleeding tendency, increased Ivy bleeding time and several other abnormalities including variable mental retardation and mild skeletal malformation. Their platelets display G_s hyper function and increased cAMP generation upon stimulation with G_s coupled receptors. $G_{s\alpha}$ gene is known to be subject to a complex pattern of genomic imprinting [366]. The variable phenotype observed among patients is explained if the resulting $G_{s\alpha}$ hyper function is co-regulated by as yet unidentified factors.

11. Defects in procoagulant expression

During regular platelet activation and secretion, surface exposure of amino phospholipids and micro vesiculation is observed. The inner leaflet of the non-activated platelet membrane contains most of the amino phospholipids, including phosphatidylethanolamine and almost all of the phosphatidylserine. The outer leaflet is enriched in neutral polar phospholipids including phosphatidylcholine and sphingomyelin [367]. As a result of activation a phospholipid 'flip-flop' results in the exposure of the phosphatidylserine on the outer layer of the membrane producing a procoagulant surface. Under normal condition the activity of the enzyme aminophospholipid 'translocase', first described in erythrocytes, maintains phosphatidylserine primarily on the inner leaflet of the platelet membrane while phosphatidylcholine is found on the outer leaflet. A slow outwardly acting phospholipid non-specific 'floppase' supposed to counterbalance the effect of translocase has been postulated [368]. It has been suggested that a third enzyme 'scramblase', isolated from erythrocytes, cloned and sequenced may be responsible for the breakdown of phospholipid asymmetry during platelet activation. The same protein is expressed in platelet and other cell lines. Activation of the scramblase appears to be reversible and dependent on a critical intracellular calcium level [369].

A deficiency in platelet procoagulant activity associated with a bleeding disorder was first described by Weiss [370,371] and is called the Scott syndrome. Scott syndrome patients present with severe bleeding disorders. Patients bleed excessively after surgical procedures and require platelet coverage. Scott syndrome platelets show (i) strongly decreased lipid scrambling (ii) decreased number of factor Va and factor VIIIa binding sites and deficiency in their ability to promote both tenase and prothrombinase activity in response to agonists (iii) impaired capacity to shed microvesicles [372]. Family studies of a second family with Scott syndrome suggested an autosomal recessive mode of inheritance [373]. Two further patients with impaired platelet procoagulant activity and prothrombinase activity have recently been identified. At least in one patient there was a decrease in α-granule factor V, which could be the basis for impaired prothrombinase activity [374].

Proteoliposomes reconstituted from Scott syndrome resulted in similar calcium induced lipid scrambling as observed for reconstitutes from normal cells, suggesting that the syndrome is caused by impaired activation of the scramblase rather than the absence of the enzyme [375].

Stormorken syndrome [376,377] is a multifaceted syndrome which presents with a moderate bleeding tendency. The most conspicuous bleeding tendency is easy bruising, and additionally, haematomas of traumatic origin, profuse bleeding during menstruation or surgery and nose bleeds. Other clinical features are asplenia, reduced platelet survival time, miosis, dyslexia, muscle fatigue and icthyosis. The most prominent of platelet deficiencies are the presence of full procoagulant activity in resting platelets (corresponds to increased annexin V binding) and the presence of high levels of microvesicles in patient plasma. The underlying cause of this abnormality is unknown.

12. Enzyme deficiencies

Patients have been described with impaired release of arachidonic acid secondary to defective phospholipase A_2 the enzyme that liberates arachidonic acid from membrane phospholipids. Patients have also been described with platelet dysfunction associated with cyclooxygenase and thromboxane synthase deficiency. The clinical presentation of the patients and platelet function abnormalities are similar to storage pool disease. Platelet function tests, in some cases, resemble those seen after aspirin ingestion [378].

13. Platelet secretion defects

These are a heterogeneous group of patients, and are characterised by a impaired secretion of granule contents and absence of second wave of aggregation on stimulation of platelet rich plasma with ADP and adrenaline. Responses to collagen, thromboxane analogues, arachidonic acid and platelet activating factor may also be impaired. Prolonged bleeding time, not always associated with reduced aggregation may also be associated with reduced platelet nucleotides and serotonin suggestive of storage pool disease [379]. The storage pool diseases are characterised into three groups by electron microscopy, decreased or abnormal α granules (α-SPD), dense granules (δ-SPD) or both (αδ-SPD).

The α-SPD or gray platelet syndrome is characterised by enlarged platelets devoid of normal alpha granule staining typically observed by the grey colour of a Wright-stained

blood smear. Family studies suggest a recessive inheritance [380,381]. Ultrastructural studies on megakaryocytes from two patients with α-SPD suggested that precursors of alpha granules are produced but their contents are then lost [382]. Other studies suggest that the fundamental defect in α-SPD may be in the targeting of endogenously synthesised secretory proteins to developing alpha granules in megakaryocytes [383]. The molecular machinery used by platelets for exocytosis, the SNARE (soluble NSF attachment protein receptor) proteins have recently been identified. Insights into the molecular mechanisms responsible for platelet exocytosis will help define some causes of human platelet secretory disorders [384].

In a report of three paediatric cases presenting with α-SPD, the older sister exhibited thrombocytopenia with recurrent ecchymoses and epistaxis since the age of 5 years. Up to the age of 2 years she also had many pulmonary infections. In these cases the megakaryocytes appeared to abnormally process vWF, with secretion into the extracellular space instead of normal α-granule packaging. In this family the secretory compartment of the neutrophil was also affected [385]. α-SPD was described in two siblings from a Bedouin family. Bone marrow aspiration and biopsy showed myelofibrosis and extensive emperipolesis. The former was attributed to the release of fibroblastic growth factors. Platelet activation was approximately normal to ADP but impaired in response to thrombin receptor activating peptide. The difference between cases suggests the need for future differentiation of various phenotypes of α-SPD [386].

The Quebec platelet disorder, is an autosomal dominant disorder, which is associated with moderate to severe delayed bleeding. The index case reported by Hayward et al. [387], experienced frequent episodes of epistaxis in childhood, bleeding for 1 month after a broken tooth, haematomas following trauma and heavy menstrual periods. Laboratory investigation reported mild thrombocytopenia, prolonged bleeding time and absent epinephrine aggregation. Platelet alpha granule contents were shown to be proteolysed [387], and associated with increased urokinase type plasminogen activator within the platelets [388]. Though the molecular genetic defects in these families is unknown, it is thought that pathologic proteolysis of alpha granular contents rather than a defect in targeting proteins to alpha granules may be the cause of protein degradation in this disorder. Moderate to severe bleeding in patients with Quebec platelet disorder can result from accelerated fibrinolysis within the haemostatic plug, where the concentration of the released plasminogen activator can overwhelm protease inhibitors [389].

The genetic disease known as collectively as Hermansky–Pudlak syndrome consists of several genetically different autosomal recessive disorders which share the clinical manifestations of hypopigmentation, platelet dense granule deficiency and accumulation of ceroid pigment in lysosomal organelles. The first reported case of Hermansky–Pudlak syndrome presented with oculocutaneous albinism and a history of frequent bruising following minimal trauma. These findings reflect abnormalities of the melanocyte's melanosome and of the platelet's dense granule. Other reported features are pulmonary fibrosis and granulomatous colitis [390]. The syndrome is common in Puerto Rico, where it is caused by mutations in the HPS1, on chromosome 19, and less often the HPS3 gene. The HPS1 gene has no recognisable function, while HPS2 disease is thought to be caused by mutations in the ADTB3A, which codes for a subunit of an adaptor complex responsible for new vesicle formation from the trans-Golgi network. HPS3 is a novel gene of unknown function. Recently, another homologue, HPS4 was identified and mutations found in non-Puerto-Rican individuals with Hermansky–Pudlak syndrome. Genes responsible for the disorder are expected to code for proteins involved in the formation, trafficking or fusion of intracellular vesicles of lysosomal lineage [391].

Another rare autosomal recessive disorder associated with clinical findings of oculocutaneous albinism and platelet storage deficiency is Chediak–Higashi syndrome. In addition Chediak–Higashi syndrome is characterised by infections and an accelerated chronic lymphohistiocytic infiltration phase. Chediak–Higashi syndrome patients have reduced or irregular platelet dense bodies, and low levels of secreted dense body constituents. Like the Hermansky–Pudlak syndrome the Chediak–Higashi syndrome results from defects in vesicles of lysosomal lineage. The only known Chediak–Higashi causing gene, LYST, codes for a large protein of unknown function. It is thought that Hermansky–Pudlak syndrome is a disease of vesicle formation and Chediak–Higashi syndrome a disease of vesicle trafficking [392,393].

Wiskott–Aldrich syndrome (WAS) and X-linked thrombocytopenia (XLT) are caused by mutations of the WAS protein (WASP) gene. The WAS phenotype consists of immunodeficiency, eczema, thrombocytopenia and is associated with extensive clinical heterogeneity. Recent work suggests that the WASP protein has a potential role in the regulation of the cytoskeleton [394]. Reports describe patients who are sometimes severely thrombocytopenic, with typically less than 10% of the normal number of circulating platelets. Their platelets are small with an effective average diameter of 1.82 ± 0.12 μm compared with a normal platelet diameter of 2.3 ± 0.12 μm. Several functional defects measured by in vitro assays have been reported in WAS platelets, however, the data obtained may reflect the reduced size or damage to platelets [395].

14. Conclusions and future directions

Recent advances in knowledge of platelet physiology suggest that the full response of platelets to vascular injury is mediated by a combination of agonists. Platelets are activated at the site of vascular injury by a combination of collagen exposure and release of soluble agonists from activated

platelets. Activation of platelets is followed by aggregation. Platelet aggregation under fluid dynamic conditions of high shear stress is mediated by adhesive ligands such as fibrinogen and vWF. Thus, the development of platelet aggregates with bonds to overcome high shear conditions requires the interaction of several ligands with their specific receptors, to mediate interplatelet cohesion. The variety of inherited disorders of platelet function reflects the complex nature of the synergistic action of several receptors with their ligands. Aggregation is enhanced by soluble agonists secreted by the platelets.

However, what is not precisely understood is the relative abilities of each of these receptors to cause platelet activation and aggregation. Several platelet receptors act in concert to cause platelet aggregation in response to vascular injury, though the underlying mechanisms, which involve one or more G-proteins are not understood. The frequency and severity of bleeding in patients may involve receptor expression, their relative abilities to cause platelet activation, and the degree to which other agonists are required to amplify the aggregation process. Recent studies also provide evidence for cross talk between receptors [396].

Polymorphisms in GP IIb–IIIa are being compared for an association with coronary events [397]. In future the analysis may be more towards a more predictable assessment of the phenotypic bleeding tendency in a patient. Patients with inherited disorders of platelet function are managed with platelet transfusion, to ensure adequate haemostatic function. Development of individualised therapy for platelet disorders requires a more exact definition of functional deficiency of platelets in an individual.

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Biography

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