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ORIGINAL ARTICLE

Molecular defects of the platelet P2 receptors

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Abstract Human platelets express three types of P2 receptors, which play important roles in platelet function: $P2X_1$, $P2Y_1$ and $P2Y_{12}$. Only patients with either quantitative or qualitative abnormalities of the platelet $P2Y_{12}$ receptor have been well-characterized so far. Deficiencies of $P2Y_{12}$ are associated with nucleotide deletions in the open-reading frame, frameshifts, and early truncation of the protein, or with a nucleotide substitution in the transduction initiation codon. Congenital dysfunctions of $P2Y_{12}$ are associated with molecular defects involving the sixth *trans*-membrane domain or the adjacent third extracellular loop of the receptor, which identify a region of the protein whose integrity is necessary for normal receptor function. A mutation, predicting a lysine to glutamate (Lys174Glu) substitution was associated with decreased ligand binding to the receptor, suggesting that it is responsible for disruption of the adenosine diphosphate (ADP)-binding site of the receptor. Patients with $P2Y_{12}$ defects display a mild-to-moderate bleeding diathesis, characterized by mucocutaneous bleedings and excessive post-surgical and post-traumatic blood loss. Defects of $P2Y_{12}$ should be suspected when ADP, even at high concentrations (≥10 μM), is unable to induce full, irreversible platelet aggregation. Tests that evaluate the degree of inhibition of adenylyl cyclase by ADP should be used to confirm the diagnosis.

Keywords Platelets · P2Y12 · P2 receptors · Adenosine diphosphate · Platelet function disorders · Platelet physiology

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Roles of P2 receptors and adenine nucleotides in platelet function

Human platelets express at least three distinct receptors stimulated by adenosine nucleotides: $P2Y_1$, $P2Y_{12}$, which bind adenosine diphosphate (ADP), and $P2X_1$, which binds adenosine triphosphate (ATP) [1]. Small amounts of a fourth P2 receptor, $P2Y_{14}$, which is activated by nanomolar and low micromolar concentrations of UDPglucose, UDP-galactose, UDP-glucuronic acid, and UDP-Nacetylglucosamine, has been detected in platelets [2, 3], but its role in platelet function is still unknown [4]

ADP, the first known low-molecular weight platelet-aggregating agent, is a weak platelet agonist: as such, it only induces shape change and reversible aggregation in human platelets, while the secretion of platelet-dense granules constituents and the ensuing secondary aggregation that are sometimes observed following stimulation with ADP of normal platelet-rich plasma (PRP) are triggered by thromboxane (Tx) A_2, whose synthesis is stimulated by platelet aggregation [5]. This phenomenon is greatly enhanced and can be observed in most individuals when the concentration of plasma Ca^{2+} is artifactually decreased to the micromolar level, such as in citrated PRP [6–8]. ADP plays a key role in platelet function because, when it is secreted from the platelet-dense granules where it is stored,

it amplifies the platelet responses induced by other platelet agonists [1, 8, 9], stabilizes platelet aggregates [10–12] and inhibits the antiplatelet effects of prostacyclin [13].

The interaction of ADP with its G_q-coupled, $P2Y_1$ receptor mediates a transient rise in cytoplasmic Ca^{2+}, platelet shape change and rapidly reversible aggregation, while its interaction with the G_i-coupled, $P2Y_{12}$ receptor mediates inhibition of adenylyl cyclase, phosphatidylinositol 3-kinase activation and the amplification of the platelet aggregation response [1]. About 20–30% of the platelet-binding sites for ADP are associated with $P2Y_1$, while the remaining 70% are associated with $P2Y_{12}$ [1, 14]. Concomitant activation of both the G_q and G_i pathways by ADP is necessary to elicit normal aggregation [1, 10].

ATP, being an antagonist of both $P2Y_1$ and $P2Y_{12}$, inhibits platelet activation by ADP [15, 16]: however, through its interaction with $P2X_1$, it can also activate platelets by inducing a very rapid influx of Ca^{2+} from the extracellular medium, which is associated with a transient platelet shape change [17]. Platelet activation by ATP amplifies the platelet responses to other agonists, especially in flow conditions that are characterized by high shear stress [18–22].

Abnormalities of the platelet P2 receptors

Abnormalities of the platelet $P2X_1$ receptors

Only one patient with defect of the platelet $P2X_1$ receptor has been described so far. She displayed a severe bleeding diathesis associated to a naturally occurring dominant negative $P2X_1$ mutant, lacking one leucine within a stretch of four leucine residues in its second transmembrane domain (amino acids 351–354) [23]. However, the patient also displayed a severe defect of ADP-induced platelet aggregation, which cannot be explained by the defect in $P2X_1$ receptor (which is activated by ATP and has no role in ADP-induced platelet aggregation) and could by itself account for the bleeding diathesis of the patient. Therefore, the relationship between genotype and phenotype in the patient described by Oury et al. is unclear.

Abnormalities of the platelet $P2Y_1$ receptors

Defects of the platelet $P2Y_1$ receptors

A description of a patient with a history of bleeding following surgery and occasional weak ADP-induced platelet aggregation was published in abstract form by Oury et al. in 1999 [24]. The defect was associated with normal $P2Y_1$-encoding regions in the patient's DNA, but reduced platelet levels of $P2Y_1$ mRNA (75% of normal), suggestive of deficient $P2Y_1$ gene transcription. Low $P2Y_1$ mRNA platelet levels were also found in a sister, son, and grandson of the index patient, but not in six other members of the family. Consistent with a deficiency of $P2Y_1$, ADP did not elicit intracellular Ca^{2+} mobilization in the index patient and his family members with low $P2Y_1$ mRNA platelet levels. However, no further details of this family have been published in a full article since this 1999 abstract.

Polymorphisms of the platelet $P2Y_1$ receptors

A $P2Y_1$ gene dimorphism, 1622AG, was associated with a significant effect on platelet ADP response, with a greater response in carriers of the G allele (frequency 0.15). The response to all tested concentrations of ADP in GG homozygotes was higher than in AA homozygotes, but greatest with 0.1 μM ADP (on average, 130% higher) [25].

Abnormalities of the platelet $P2Y_{12}$ receptors

Congenital deficiency of $P2Y_{12}$

Congenital $P2Y_{12}$ deficiency is an autosomal recessive disorder. The first patient with congenital severe $P2Y_{12}$ deficiency (VR), with lifelong history of excessive bleeding and prolonged bleeding time (15–20 min), was described in 1992 [26]. The most typical abnormality of platelet function observed in this patient was that ADP, even at very high concentrations (>10 μM), did not induce full and irreversible platelet aggregation. Other abnormalities of platelet function were: (a) reversible aggregation in response to weak agonists and impaired aggregation in response to low concentrations of collagen or thrombin, due to the lack of the amplification of platelet responses mediated by the interaction of ADP, secreted by platelet granules, with $P2Y_{12}$; (b) no inhibition by ADP of prostaglandin E_1 (PGE_1)-stimulated platelet adenylyl cyclase, but normal inhibition by epinephrine; (c) normal shape change and borderline-normal mobilization of cytoplasmic Ca^{2+} induced by ADP; (d) presence of approximately 30% of the normal number of binding sites for [^{33}P]2 methylthioadenosine diphosphate (2MeSADP) on fresh platelets [27] or [^{3}H]ADP on formalin-fixed platelets [26]. Five additional patients with severe $P2Y_{12}$ deficiency, belonging to four kindreds, were subsequently described: one French man (ML) [28], two Italian sisters (IG and MG) [29] a Japanese woman (OSP-1) [30], and a British woman of Asian descent [31] (Table 1).

Heterozygous $P2Y_{12}$ deficiency is characterized by [29]: reversible platelet aggregation induced by ADP concentrations ≤10 μM; full and irreversible platelet aggregation induced by concentrations of ADP ≥10 μM; impaired, but

Table 1 Characteristics of the patients with congenital $P2Y_{12}$ deficiency

Patient identification [reference]	$P2Y_{12}$ mutations	Binding sites for 2MeS-ADP	Platelet aggregation induced by ADP≥10 μM	Bleeding time (min)	History of abnormal bleeding
VR [26, 34]	p.[Gln98fs]+[Gln98fs]	Severely reduced	Reduced and reversible	15; 20	Yes
ML [28, 36]	p.[Phe240fs]+[?][a]	Severely reduced	Reduced and reversible	>20	Yes
IG [29, 35]	p.[0]+p.Thr126fs[c]	Severely reduced	Reduced and reversible	>30	Yes
MG [29, 35]	p.[0]+p.Thr126fs[c]	Severely reduced	Reduced and reversible	20	Yes
OSP-1 [30]	p.[0]+[0][b]	NA	Reduced and reversible	>15	Yes
?? [31]	p.[Gly12fs]+[Gly12fs]	NA	Reduced and reversible	NA	Yes
CL [28, 36]	p.[Phe240fs]+[=]	Intermediate	Full and irreversible	NA	No
GL [29, 35]	p.[0]+[=][c]	Intermediate	Full and irreversible	13	No

Patient CL is the daughter of patient ML; patient GL is the son of patient MG who is the sister of patient IG

The upper normal limit of the bleeding time varies between 8 and 10 min in different laboratories

NA not available

[a] No mutations were found in one allele of patient ML; however, the findings that the patient's platelets contained $P2Y_{12}$ transcripts derived from the mutant allele only and that his daughter (CL) inherited the mutant allele from her father and a normal allele from her mother, suggest that patient ML has an additional, as yet unknown mutation that silences his normal allele (see text for details)

[b] Failure of expression of the $P2Y_{12}$ protein (p.[0]) in patient OSP-1 was associated with homozygous single nucleotide substitution in the transduction initiation codon (ATG to AGG)

[c] p[0] was associated with partial or complete $P2Y_{12}$ gene deletion in patients IG, MG and GL

not absent inhibition of PGE_1-induced increase in platelet cyclic AMP; impaired platelet secretion induced by several agonists. Because the platelet secretion defect is not associated with impaired production of TxA_2 or low concentrations of platelet granule contents, it is very similar to that described in patients with an ill-defined and probably heterogeneous group of congenital defect of platelet secretion, sometimes referred to with the general term "primary secretion defect" [32, 33].

The $P2Y_{12}$ gene of patient VR and of the British patient with Asian descent displayed homozygous base pair deletions in the open-reading frame, resulting in frameshifts and premature truncation of the protein [31, 34]. Patients IG and MG suffer from $P2Y_{12}$ deficiency, owing to haploinsufficiency [35] and to a 378delC mutation in their remaining allele, which results in a frameshift and premature truncation of the protein [34]. Patient OSP-1 is homozygous for a single nucleotide substitution in the transduction initiation codon (ATG to AGG) [30]. The molecular defect that is responsible for $P2Y_{12}$ deficiency in patient ML [28] is less well defined [36]: one mutant allele contains a deletion of two base pairs, resulting in a frameshift and early truncation of the protein. Surprisingly, the other allele did not display any mutation: the findings that the patient's platelets contained $P2Y_{12}$ transcripts derived from the mutant allele only and that his daughter, who had a heterozygous phenotype, inherited the mutant allele from her father and a normal allele from her mother, suggest that patient ML has an additional, as yet unknown mutation that silences his normal allele.

Congenital dysfunction of the platelet $P2Y_{12}$ receptors

A patient (AC) with dysfunstional $P2Y_{12}$ was described in 2003 [37]. His bleeding disorder was associated with normal binding of [^{33}P]2MeSADP to platelets, but abnormal $P2Y_{12}$-mediated platelet responses to ADP: (a) similar to platelets with severe deficiency of $P2Y_{12}$, platelet aggregation induced by 4 μM ADP was low and reversible, but the response to 20 μM ADP, albeit still decreased and reversible, was more pronounced and was further inhibited by a $P2Y_{12}$ antagonist, indicating residual receptor function; (b) ADP failed to lower adenylyl cyclase activity stimulated by PGE_1. Analysis of the patient's $P2Y_{12}$ gene revealed, in one allele, a G to A transition changing the codon for Arg256 in sixth *trans*-membrane domain (TM6) to Gln and, in the other, a C to T transition changing the codon for Arg265 in the third extracellular loop (EL3) to Trp (Table 2). Neither mutation interfered with receptor surface expression but both altered receptor function, since ADP inhibited the forskolin-induced increase of cyclic AMP markedly less in cells transfected with either mutant $P2Y_{12}$ than in wild-type cells. These observations, in accordance with previous studies of the $P2Y_1$ receptor [38, 39], helped to identify regions in TM6 and EL3, whose structural integrity is necessary for normal receptor function. A heterozygous point mutation in the same region of the molecule, which changed codon 258 coding for proline (CCT) to threonine (ACT) (Pro258Thr), was described in a patient with mild bleeding disorder and severely impaired ADP-induced platelet aggregation [40]. Since the proline to

Table 2 Characteristics of the patients with congenital dysfunction of $P2Y_{12}$

Patient identification [reference]	$P2Y_{12}$ mutations	Binding sites for 2MeS-ADP	Platelet aggregation induced by ADP≥10 μM	Bleeding time (min)	History of abnormal bleeding
AC [37]	p.[Arg256Gln]+[Arg265Trp]	Normal	Reduced and reversible	19; 20	Yes
MC [37]	p.[Arg265Trp]+[=]	Normal	Full and irreversible	7	No
FC [37]	p.[Arg265Trp]+[=]	Normal	Full and irreversible	6.5	No
GS [40]	p.[Pro258Thr]+[=]	NA	Reduced and reversible	Normal/slightly prolonged	Yes
PII.1 [41]	p.[Lys174Glu]+[=]	Intermediate	Reduced and reversible	NA	Yes[a]

NA not available

Patients MC and FC are the son and the daughter of patient AC

The upper normal limit of the Bleeding Time varies between 8 and 10 min in different laboratories

[a] Patient PII.1 also had type-1 von Willebrand Disease

threonine substitution alters the protein hydrophobicity, size, and rotational mobility, it is likely to affect the function of $P2Y_{12}$.

A heterozygous mutation, predicting a lysine to glutamate (Lys174Glu) substitution in $P2Y_{12}$, was identified in one patient with mild type 1 von Willebrand disease [41]. Platelets from this patient showed reduced and reversible aggregation in response to ADP, up to 10 μM. The reduced response was associated with an approximate 50% reduction in binding of [^{3}H]2MeS-ADP. Considering that Lys174 is situated in the second extracellular loop of $P2Y_{12}$, adjacent to Cys175, which is linked to a Cys at position 97 residue and may be important for the expression of the ADP binding site receptor [42–45], and that a hemagglutinin-tagged Lys174Glu $P2Y_{12}$ variant showed surface expression in Chinese hamster ovary cells, it is likely that the Lys174Glu mutation is responsible for disruption of the ADP binding site of the receptor.

For reasons that are presently unclear, two patients with heterozygous dysfunctional $P2Y_{12}$ (Pro258Thr and Lys174Glu) display a much more severe impairment of ADP-induced platelet aggregation compared to the two patients who are heterozygous for $P2Y_{12}$ deficiency [28, 29] and to the two children of patient AC, who are heterozygous for the Arg265Gln mutation [37] (Table 2).

Clinical features of congenital P2Y12 defects

Bleeding manifestations

Patients with defects of $P2Y_{12}$ experience mucocutaneous bleeding and excessive post-surgical or post-traumatic blood loss. The severity of their bleeding diathesis is variable. The bleeding scores of patient VR and of the two sisters, MG and IG, which was calculated using a standardized questionnaire that was developed to investigate patients with type-1 von Willebrand disease [46] were 8, 7, and 13, respectively, (normal values ≤3; unpublished data). The degree of prolongation of their bleeding times was also variable, reflecting the severity of their clinical bleeding scores: 15 and 20 min (results of two measurements in patient VR), 20 min (patient MG) and >30 min (patient IG; normal values <8 min). After extensive investigation of haemostasis parameters, which included measurement of the activity of clotting and fibrinolytic factors and the search for known polymorphisms of haemostasis proteins, we found no explanation for the discrepancy in the severity of bleeding manifestations in the two sisters, MG and IG.

The bleeding score of a patient with heterozygous $P2Y_{12}$ deficiency (GL, the son of patient MG) was normal; however, it must be noted that this young boy had not yet experienced situations that could challenge the haemostatic system at the time of our investigation. His bleeding time, despite the mild defect of $P2Y_{12}$, was prolonged (13 min).

Diagnosis

The diagnosis of $P2Y_{12}$ defects is rather simple: they should be suspected when ADP, even at relatively high concentrations (≥10 μM), is unable to induce full, irreversible platelet aggregation, while inducing normal shape change. Tests that evaluate the degree of inhibition of adenylyl cyclase by ADP, by measuring either the platelet levels of cyclic AMP or the phosphorylation of vasodilator-stimulated phosphoprotein [47] after the exposure of platelets to PGE_1, should be used to confirm the diagnosis.

Treatment

The intravenous infusion of the vasopressin analog desmopressin (0.3 μg/kg) shortened the prolonged bleeding time of patient VR from 20 to 8.5 min [48]. After the infusion of desmopressin, which was repeated twice at 24 h intervals,

the patient underwent a surgical intervention for disk hernia repair, which was not complicated by excessive bleeding. Although the efficacy of desmopressin in reducing bleeding complications of patients with defects of primary haemostasis is anecdotal [49], its administration is generally without serious side effects.

Polymorphisms of the $P2Y_{12}$ gene

Four polymorphisms of the $P2Y_{12}$ gene were identified, which were in total linkage disequilibrium, determining haplotypes H1 and H2, with respective allelic frequencies of 0.86 and 0.14. H2 haplotype is a gain-of-function haplotype, associated with increased ADP-induced platelet aggregation [50]. The H2 haplotype was more frequent among 184 patients with peripheral artery disease than in 330 age-matched control subjects (OR, 2.3; CI, 1.4–3.9; p= 0.002 after adjustment for diabetes, smoking, hypertension, hypercholesterolemia, and other selected platelet receptor gene polymorphisms) [51].

Several studies tested the hypothesis that common polymorphisms of the $P2Y_{12}$ gene might interfere with the antiplatelet effects of drugs inhibiting the $P2Y_{12}$ receptor, accounting for the well known individual variability in the response to these agents [52, 53]. The vast majority of these studies, with few exceptions, reported that $P2Y_{12}$ polymorphisms are not associated with altered platelet function inhibition by $P2Y_{12}$ antagonists [54–61].

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