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Brief report

Identification and characterization of a novel $P2Y_{12}$ variant in a patient diagnosed with type 1 von Willebrand disease in the European MCMDM-1VWD study

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We investigated whether defects in the $P2Y_{12}$ ADP receptor gene *(P2RY12)* contribute to the bleeding tendency in 92 index cases enrolled in the European MCMDM-1VWD study. A heterozygous mutation, predicting a lysine to glutamate (K174E) substitution in $P2Y_{12}$, was identified in one case with mild type 1 von Willebrand disease (VWD) and a *VWF* defect. Platelets from the index case and relatives carrying the K174E defect changed shape in response to ADP, but showed reduced and reversible aggregation in response to 10 μM ADP, unlike the maximal, sustained aggregation observed in controls. The reduced response was associated with an approximate 50% reduction in binding of [^{3}H]2MeS-ADP to $P2Y_{12}$, whereas binding to the $P2Y_1$ receptor was normal. A hemagglutinin-tagged K174E $P2Y_{12}$ variant showed surface expression in CHO cells, markedly reduced binding to [^{3}H]2MeS-ADP, and minimal ADP-mediated inhibition of forskolin-induced adenylyl cyclase activity. Our results provide further evidence for locus heterogeneity in type 1 VWD. (Blood. 2009;113:4110-4113)

Introduction

Data from the European Molecular and Clinical Markers for the Diagnosis and Management of type 1 von Willebrand Disease (MCMDM-1VWD) study, and the Canadian Type 1 VWD Study, suggest locus heterogeneity contributes to the pathogenesis of type 1 VWD, though, as yet, no associated defect in platelet reactivity has been described.[1,2] Given the role of von Willebrand factor (VWF) in primary hemostasis, and the clinical similarities of patients with type 1 VWD and platelet-based bleeding disorders, the bleeding tendency in patients with type 1 VWD may be influenced by defects in the receptor or signaling proteins mediating platelet adhesion and aggregation.

ADP is a feedback mediator of platelet aggregation, which elicits its response through the G protein–coupled receptors, $P2Y_1$ and $P2Y_{12}$. Interaction of ADP with $P2Y_1$ leads to mobilization of intracellular calcium and activation of Rho kinase, resulting in platelet shape change and an initial wave of rapidly reversible aggregation.[3-5] In contrast, interaction with $P2Y_{12}$ is associated with adenylyl cyclase inhibition and PI3-kinase activation, resulting in sustained aggregation in synergy with the $P2Y_1$ receptor.[5] Both receptors are required for a full aggregation response to ADP.[6]

We investigated the possible contribution of $P2Y_{12}$ gene *(P2RY12)* defects to the bleeding tendency in type 1 VWD patients recruited through the MCMDM-1VWD study.

Methods

Study design and evaluation of bleeding

The phenotypic and genotypic characteristics of the MCMDM-1VWD cohort have been described previously.[1] This study focused on those index cases having normal VWF multimer profiles, usually considered a criterion for type 1 VWD. This study has received local ethics committee approval at each of the centers that participated in the MCMDM-1VWD study, and informed consent was obtained in accordance with the Declaration of Helsinki.

Genetic studies

P2RY12 coding sequences were amplified from genomic DNA and sequenced on an ABI 3730 DNA capillary sequencer. The *P2RY12* 520A>G mutation was sought in other subjects by restriction analysis using *Bsm*I.

Platelet function studies

Platelet aggregation and ATP secretion were assessed in platelet-rich plasma (PRP) using a dual Chronolog lumiaggregometer (Havertown, PA) as previously described.[7]

Ligand binding studies in platelets

Ligand binding studies using [^{3}H]2MeS-ADP (3 Ci [111 GBq]/mmol) were performed as previously described.[8]

$P2Y_{12}$ constructs, cell culture, and transfections

A $P2Y_{12}$ cDNA clone for the K174E variant was derived by amplification from a hemagglutinin (HA)–tagged $P2Y_{12}$ construct.[9] CHO-K1 cells stably expressing either wild-type or K174E variant construct were generated as previously described.[9] Surface expression and cellular distribution of HA-tagged receptors were assessed in transfected cells by enzyme-linked immunosorbent assay (ELISA) and immunofluorescence microscopy as described previously.[10]

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Assessment of cAMP accumulation in CHO cells

ADP-induced inhibition of forskolin (1 μM)–stimulated cAMP accumulation in CHO cells was performed as previously described.[9,11] Data were expressed as percentage inhibition of forskolin-stimulated adenylyl cyclase.

Radioligand binding in CHO cells

Cells expressing receptor constructs were harvested and incubated with [^{3}H]2MeS-ADP (3 Ci [111 GBq]/mmol) and binding was determined in the presence of AR-C69931MX (10 μM) as described previously.[8]

Results and discussion

Identification of the K174E mutation

P2RY12 analysis, in DNA from 92 index cases enrolled in the MCMDM-1VWD study with normal VWF multimers, identified a heterozygous 520A>G transition in one case, P12F13II.1, referred to hereafter as PII.1. The transition, which predicts substitution of lysine 174 by glutamate (K174E) in $P2Y_{12}$, also occurred in several relatives of PII.1, but not in 80 control subjects from the same center as PII.1, suggesting it was unlikely to represent a polymorphism.

Platelet activation in carriers of the K174E mutation

Platelets from 3 carriers of the K174E mutation, PII.1, her mother (PI.1), and her sister (PII.2), changed shape in response to ADP in the concentration range of 3 to 30 μM, indicating normal $P2Y_1$ receptor function (Figure 1A). However, platelets from all 3 patients exhibited reduced and transient aggregation to a concentration of ADP (10 μM) that induced maximal sustained aggregation of platelets from more than 40 control subjects.[7] There was also a reduced response to 3 μM ADP, whereas 30 μM ADP induced maximal, sustained aggregation similar to that in controls (Figure 1A). These results indicated a partial defect in the $P2Y_{12}$ receptor.[7] This was supported by the further reduction in ADP (10 μM)–induced platelet aggregation caused by the $P2Y_{12}$ receptor antagonist, ARC-67085 (Figure 1B), and by the reduction in the maximal level of dense granule secretion induced by ADP in platelets from the 2 patients in whom this was measured (Figure 1C). Thus, heterozygous expression of the K174E mutation induces a partial loss in aggregation and secretion to ADP.

Aggregation of platelets from PII.1 and PI.1 in response to other agonists was also examined. Platelets from both subjects displayed sustained aggregation, similar to that in controls, in response to high concentrations of the GPVI agonist collagen-related peptide (CRP; 1 μg/mL), collagen (3 μg/mL), PAR-1 (100 μM)– and PAR-4–specific peptides (500 μM), and the thromboxane A_2 analog U46619 (3 μM) (not shown). However, reduced aggregation to lower concentrations of these agonists, consistent with a positive feedback role for ADP, was observed (not shown). Further, a reduction of approximately 50% in platelet ATP secretion was observed in response to a single high concentration of ADP and the PAR-4 peptide, reflecting the critical feedback role of ADP in this response (Figure 1C).

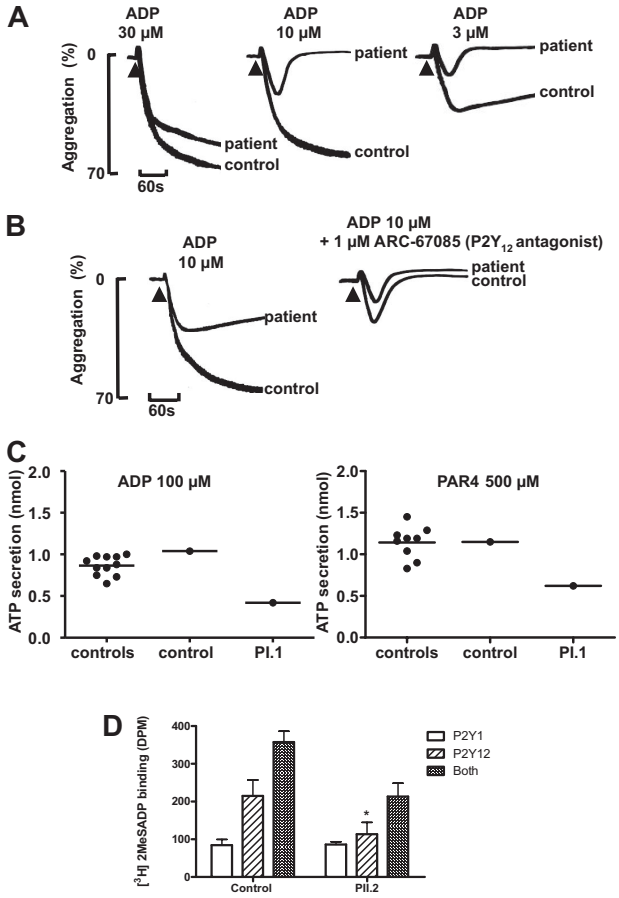

Figure 1. Agonist-induced platelet activation and [^{3}H]2MeS-ADP binding in heterozygous carriers of the $P2Y_{12}$ K174E mutation. (A) Platelet aggregation in response to the indicated concentrations of ADP in citrated PRP from a healthy volunteer (control) and from PII.2 (patient). (B) Aggregation of platelets in PRP from a healthy volunteer (control) and PI.1 (patient) in response to 10 μM ADP in the absence and presence of the $P2Y_{12}$ antagonist ARC-67085 (1 μM) as shown. (C) Maximal levels of ATP secretion in PRP from a control subject and PI.1 induced by ADP (100 μM) and a PAR-4 peptide (500 μM) as indicated. The maximal level of secretion in each sample is compared with the maximal levels of ATP secretion in PRP from control subjects studied on separate occasions. (D) $P2Y_1$ and $P2Y_{12}$ surface receptor levels were measured in fixed platelets by displacement of [^{3}H]2MeS-ADP (100 nM) by receptor antagonists for $P2Y_1$ (A3P5P; 1 mM) and $P2Y_{12}$ (AR-C69931MX; 1 μM), respectively. Data are expressed as [^{3}H]2MeS-ADP binding (DPM) and represent means (± SEM) of 3 independent experiments. *Statistically significant reduction in $P2Y_{12}$ binding levels at $P < .05$ for data compared with respective control data (Mann-Whitney *U* test).

[^{3}H]2MeS-ADP binding to platelets from carriers of the K174E mutation

K174 is situated in the second extracellular loop of $P2Y_{12}$, adjacent to cysteine 175, 1 of 4 cysteine residues in $P2Y_{12}$ predicted to be exposed on the cell surface and likely to be involved in binding ADP.[12,13] Its substitution by glutamate was therefore likely to inhibit ADP binding. Consistent with this, [^{3}H]2MeS-ADP binding to $P2Y_{12}$ on platelets from carriers of the K174E mutation was reduced by up to 50%, whereas binding to $P2Y_1$ was similar to that on control platelets (Figure 1D).

Heterologous expression of wild-type and variant $P2Y_{12}$ receptors

The defect associated with the K174E substitution was further investigated in CHO cells stably expressing HA-tagged wild-type and variant K174E $P2Y_{12}$ receptors. The wild-type and variant

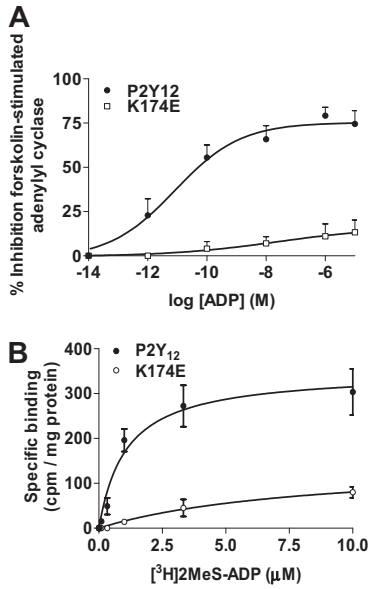

Figure 2. Markedly impaired signaling and binding to $P2Y_{12}$ K174E. (A) Agonist (ADP; 0.01 pM to 10 μM)–dependent inhibition of forskolin (1 μM; 10 minutes)–stimulated adenylyl cyclase activity was assessed in CHO cells stably expressing wild-type and K174E $P2Y_{12}$ receptor. Data are expressed as percentage inhibition of forskolin-stimulated adenylyl cyclase and represent means (± SEM) of 3 independent experiments. (B) Receptor levels were measured in CHO cells stably expressing wild-type or K174E receptor using [^{3}H]2MeS-ADP (0.1-10 μM) in the presence of the $P2Y_{12}$ receptor antagonist AR-C69931MX (1 μM) to determine specific binding. Data are expressed as specific binding of [^{3}H]2MeS-ADP (cpm) per milligram of protein and represent means (± SEM) of 3 independent experiments.

K174E receptors were expressed at similar levels on the surface of CHO cells as assessed by ELISA and immunofluorescence,[9] indicating that the mutation did not disrupt receptor synthesis or trafficking (not shown). However, the K174E variant demonstrated a marked reduction in its ability to negatively couple to adenylyl cyclase as evidenced by the decrease in both maximal response and rightward shift of the concentration-response curve (Figure 2A). The disruption in ligand binding caused by the K174E substitution in platelets was confirmed in the transfected CHO cells using [^{3}H]2MeS-ADP. Thus, whereas binding to CHO cells expressing the wild-type receptor was saturable and maximal at a ligand concentration of 1 μM (K_d 0.31 ± 0.05 μM), the K174E variant demonstrated approximately 25% of the ligand binding capacity of wild-type $P2Y_{12}$ at a ligand concentration of up to 10 μM (Figure 2B). Therefore, defective ADP-dependent inhibition of forskolin-stimulated adenylyl cyclase activity in cells expressing the K174E variant occurred as a result of a failure in ligand binding.

Association of the K174E mutation with bleeding

The mild bleeding associated with $P2Y_{12}$ deficiency is thought to be recessively inherited. Whereas this has been true for 6 of the 7 $P2Y_{12}$-deficient cases reported to date,[14-19] one patient with a hemorrhagic diathesis and a heterozygous *P2RY12* mutation (P258T) has been described, whose platelets failed to aggregate in response to low concentrations of ADP (≤ 4 mM), but showed reduced and reversible aggregation at higher concentrations of ADP (20 μM). This is similar to the profile observed in the 3 patients who have been investigated in this study.[20]

In conclusion, the identification of a novel $P2Y_{12}$ defect and its association with bleeding in a family with type 1 VWD supports a contribution from other loci to the bleeding tendency in patients diagnosed with type 1 VWD, providing further evidence for locus heterogeneity in this disorder.

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Authorship

Contribution: S.P.W. coordinated the study, which was initiated by M.E.D., A.D.M., M.M., J.T.W., I.R.P., and S.P.W.; M.E.D., S.P.W., S.J.M., and B.B.D. wrote the paper, which was read and commented on by all authors; M.E.D., B.B.D., S.J.M., W.A.L., and S.P.W. participated in the data collection and laboratory analyses; and A.C.G., I.R.P., and F.R. initiated and coordinated the MCMDM-1VWD study.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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