Brief report

Identification and characterization of a novel P2Y12 variant in a patient diagnosed with type 1 von Willebrand disease in the European MCMDM-1VWD study

Martina E. Daly,1 Ban B. Dawood,2 William A. Lester,3 Ian R. Peake,1 Francesco Rodeghiero,4 Anne C. Goodeve,1 Michael Makris,1 Jonathan T. Wilde,3 Andrew D. Mumford,5 Stephen P. Watson,2 and Stuart J. Mundell6

1Academic Unit of Haematology, School of Medicine and Biomedical Sciences, University of Sheffield, Sheffield, United Kingdom; 2Centre for Cardiovascular Sciences, University of Birmingham, Birmingham, United Kingdom; 3West Midlands Adult Haemophilia Centre, University Hospital Birmingham National Health Service (NHS) Trust, Birmingham, United Kingdom; 4Hematology Department, San Bortolo Hospital, Vicenza, Italy; 5Bristol Heart Institute, University of Bristol, Bristol Royal Infirmary, Bristol, United Kingdom; and 6Department of Pharmacology, University of Bristol, Bristol, United Kingdom

We investigated whether defects in the P2Y12 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 P2Y12, 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 [3H]2MeS-ADP to P2Y12, whereas binding to the P2Y1 receptor was normal. A hemagglutinin-tagged K174E P2Y12 variant showed surface expression in CHO cells, markedly reduced binding to [3H]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, P2Y1 and P2Y12. Interaction of ADP with P2Y1 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 P2Y12 is associated with adenylyl cyclase inhibition and PI3-kinase activation, resulting in sustained aggregation in synergy with the P2Y1 receptor.6 Both receptors are required for a full aggregation response to ADP.6

We investigated the possible contribution of P2Y12 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 BsmI.

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 [3H]2MeS-ADP (3 Ci [111 GBq]/mmol) were performed as previously described.8

P2Y12 constructs, cell culture, and transfections

A P2Y12 cDNA clone for the K174E variant was derived by amplification from a hemagglutinin (HA)-tagged P2Y12 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
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. 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 [3H]2MeS-ADP (3 Ci [111 GBq]/mmol) and binding was determined in the presence of AR-C69931MX (10 μM) as described previously.

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, P12F13IL1, referred to hereafter as PI.1. The transition, which predicts substitution of lysine 174 by glutamate (K174E) in P2Y12, also occurred in several relatives of PI.1, but not in 80 control subjects from the same center as PI.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, PI.1, her mother (PI.1), and her sister (PII.2), changed shape in response to hereafter as PII.1. The transition, which predicts substitution of cysteine 175, 1 of 4 cysteine residues in P2Y12 predicted to be critical feedback role of ADP in this response (Figure 1C).

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

K174 is situated in the second extracellular loop of P2Y12, adjacent to cysteine 175, 1 of 4 cysteine residues in P2Y12 predicted to be exposed on the cell surface and likely to be involved in binding ADP. Its substitution by glutamate was therefore likely to inhibit ADP binding. Consistent with this, [3H]2MeS-ADP binding to P2Y12 on platelets from carriers of the K174E mutation was reduced by up to 50%, whereas binding to P2Y1 was similar to that on control platelets (Figure 1D).

Heterologous expression of wild-type and variant P2Y12 receptors

The defect associated with the K174E substitution was further investigated in CHO cells stably expressing HA-tagged wild-type and variant K174E P2Y12 receptors. The wild-type and variant
Association of the K174E mutation with bleeding

The mild bleeding associated with P2Y₁₂ deficiency is thought to be recessively inherited. Whereas this has been true for 6 of the 7 P2Y₁₂-deficient cases reported to date,¹⁴-¹⁵ 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 nM), 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 P2Y₁₂ 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.

Acknowledgments

The contribution of the MCMDM-1VWD study partnership is gratefully acknowledged. We thank Mr John Anson for his contribution to the laboratory analyses.

This work was supported by the British Heart Foundation (BHF; PG/06/038). The MCMDM-1VWD study was supported by the European Community under the Fifth Framework Program (QLG1-CT-2000-00387). S.P.W. holds a BHF Senior Lectureship. S.P.W. holds a BHF Chair.

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.

Correspondence: Martina Daly, Academic Unit of Haematology, School of Medicine and Biomedical Sciences, Beech Hill Road, Sheffield, S10 2RX, United Kingdom; e-mail: m.daly@sheffield.ac.uk.

References

11. Mundell SJ, Benovic JL, Kelly E. Selective inhibition of adenosine A2 receptor desensitization in NG108-15 cells stably transfected with dominant...


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