bjh short report

Bleeding disorders in Lowe syndrome patients: evidence for a link between *OCRL* mutations and primary haemostasis disorders

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Summary

Lowe syndrome (LS) is a rare X-linked disorder caused by mutations in the oculocerebrorenal gene (*OCRL*), encoding OCRL, a phosphatidylinositol 5–phosphatase with a RhoGAP domain. An abnormal rate of haemorrhagic events was found in a retrospective clinical survey. Herein, we report the results of exploration of haemostasis in six LS patients. All patients had normal coagulation tests but prolonged closure times (CTs) in the PFA–100 system. Healthy donors' blood samples incubated with a RhoA kinase inhibitor had prolonged CTs. This suggests that an aberrant RhoA pathway in platelets contributes to CT prolongation and primary haemostasis disorders in LS.

Keywords: Lowe syndrome, OCRL, RhoGAP, platelets, haemostasis disorders.

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Lowe syndrome (LS), also known as oculocerebrorenal syndrome of Lowe (OCRL), is a rare X-linked condition characterized by congenital cataracts, defective renal tubule cell function, muscular hypotonia and variable degrees of mental retardation. Patients with LS require frequent surgery, some of which are associated with a severe haemorrhagic risk, such as scoliosis reduction, hip surgery, or eye surgery. In a recent retrospective clinical survey of French LS patients, we observed an abnormal rate of haemorrhagic events, some of which had dramatic outcomes (Baujat, G. unpublished data). Based on that clinical observation, particular attention is now paid to bleeding-risk evaluations for these patients, especially before surgery. LS is caused by mutations in the *OCRL* gene, which encodes OCRL, an inositol polyphosphate 5-phosphatase. The preferred OCRL substrate is the membrane phospholipid phosphatidylinositol-4,5-bisphosphate ($PtdIns(4,5)P_2$) (Attree *et al*, 1992). OCRL also contains a Rho GTPase-activating protein (GAP)-like domain that participates in the regulation of Rho proteins (Rho, Rac, Cdc42), as GTPase-activating proteins or by mediating in protein–protein interactions. $PtdIns(4,5)P_2$ and Rho-dependent signalling play a central role in many important cellular processes, including vesicular trafficking and cytoskeletal organization (Raucher *et al*, 2000; Ridley, 2001), both of which are very important for platelet function. Thus, modulation of $PtdIns(4,5)P_2$ levels and/or Rho-dependent signalling would be expected to impact platelet function.

Herein, we report the results of systematic exploration of haemostasis in LS patients.

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Design and methods

Patients

This study included six consecutive unrelated male juveniles admitted to our Paediatric Nephrology Unit who fulfilled the following diagnosis criteria for LS: bilateral congenital cataracts, proximal tubulopathy and mental retardation. *OCRL* was screened for mutations by single-strand conformation analysis (SSCA) sequencing techniques as previously described (Satre *et al*, 1999), after informed consent was obtained from all patients or their parents, in accordance with French law. A medical questionnaire to obtain information on previous haemorrhagic events, during surgery or trauma, or spontaneous was completed for each patient.

Laboratory tests

Haemostasis was explored with clotting tests (prothrombin time (PT), activated partial thromboplastin time (aPTT), and fibrinogen level), on 0·129 mol/l citrated blood using the STA analyzer (Diagnostica Stago, Asnières, France). We also explored primary haemostasis with the platelet-function analyzer (PFA-100 system, Siemens, Marburg, Germany). Blood specimens were analysed within 1 h of sampling. Platelet-poor-plasma was stored at −80°C until tested for von Willebrand factor (VWF) antigen (Liatest VWF: Ag, Diagnostica Stago) and VWF ristocetin cofactor activity performed by optical aggregometry (Regulest Aggregometer; AFFI-Bio, Nancy, France). EDTA-anticoagulated blood was used for haematocrit determination and blood cell counts on Sysmex XE-2100 (Sysmex Corporation, Kobe, Japan).

Platelet functions were assessed with light transmission aggregometry in platelet-rich plasma (PRP) (Regulest Aggregometer; AFFI-Bio) as recommended for the diagnosis of bleeding disorders (Hayward *et al*, 2009) and with flow cytometry on FACScan flow cytometer and CellQuest software (Becton Dickinson, San Jose, USA) using platelet Gp/receptors kit (Biocytex, Marseille, France).

ROCK, a RhoA-activated kinase, was inhibited with Y27632 (Sigma Aldrich, St Louis, MO, USA). Adenosine 5-diphosphate (ADP) closure times (CTs) were measured after a 60-s preincubation of citrated blood from seven control subjects (healthy blood donors from the Etablissement Français du Sang, enrolled with their written informed consent) with 200 μmol/l Y27632 or vehicle (0·9% NaCl). In the same way, platelet aggregation induced by thrombin-receptor-activating-peptide (TRAP) (Bachem, Budendorf, Switzerland) was studied after preincubation of PRP with Y27632 or vehicle.

Results and discussion

Patients' mutations, demographics and clinical characteristics are given in Table I. Their median [range] age was 7·5 [1–17] years. Four patients reported a bleeding history: Patients 1

Table I. Demographic, clinical and biological characteristics of six Lowe syndrome patients.

Patient	Age (years)	Mutation*	Previous surgery	Haemorrhagic complication	PFA-100 Closure Times		von Willebrand factor		Haemoglobin	Fibrinogen	Platelets
					EPI	ADP	Antigen	Ristocetin Cofactor Activity			
					(85–165 s)†	(71–118 s)†	(50–150%)†	(50–150%)†	(>105 g/l)†	(2–4 g/l)†	(>175 × 10^9/l)*
1	1	c.2469+2 T>G	Eye	Yes	**181**	**135**	**279**	**216**	**102**	**8·2**	267
2	10	p.Arg663X	Neurological & eye	Yes	**>300**	**166**	**305**	**199**	**98**	**5·1**	178
3	5	del *OCRL*	Eye	No	**195**	**126**	**295**	**275**	117	**5·2**	336
4	2	p.Gly421Glu	Eye	Yes	**197**	**128**	**251**	**200**	**101**	3·5	195
5	17	p.Cys498Tyr	Tooth extraction	No	**>300**	**129**	**262**	**200**	117	4	**143**
6	14	p.Arg500Gly	Tooth extraction	Yes	**189**	**188**	**332**	**224**	113	2·9	**112**

*According to the nomenclature described by Erdmann *et al* (2007).
†Normal range for individuals older than 1 year.
Abnormal values are given in bold type.

and 4 suffered from haemorrhagic complications of cataract surgery during infancy. Patient 6 had experienced severe late bleeding after tooth extraction at 14 years. Patient 2 had 2 bleeding events, during cataract extraction, and during surgery for craniostenosis at 3 years. Although transfusion is not uncommon during craniostenosis surgery, the prolonged bleeding in Patient 2 was unusual.

PT and aPTT were normal in all patients (13·06 ± 0·67 s, normal value <14 s and aPTT ratio 0·99 ± 0·1, normal value <1·2, respectively). Fibrinogen level was elevated in three patients and borderline high in a fourth.

Interestingly, PFA-100 CT was abnormal for all six patients (Table I). The PFA-100 simulates primary haemostasis after injury to a small vessel (Lasne *et al*, 2000). It measures the time necessary to obtain a full occlusion of a membrane aperture under high-shear, which is dependent on platelet activation, adhesion and aggregation. In LS patients, CTs were prolonged with both cartridges, coated with collagen, and either epinephrine (CEPI), or ADP (CADP). The PFA-100 is sensitive to low platelet counts, low haemoglobin concentrations, VWF defects and platelet dysfunctions (Lasne *et al*, 2000). Although haemoglobin concentrations were below the normal value for three patients, it exceeded 98 g/l in all patients, excluding a major impact of anaemia on PFA results. Similarly, a low platelet count could not explain abnormal CTs, as all patients' counts were over 100×10^9/l. Moreover, these prolonged CTs were not associated with diminished VWF (Table I). Furthermore, an abnormality in the multimeric structure of plasma VWF was excluded by electrophoresis analysis (data not shown).

Hence, the isolated PFA abnormal results suggested platelet abnormalities in LS patients. However, platelet aggregation and glycoprotein quantification (CD41a (αIIbβ3), CD42b (GPIb) and CD62P (p-selectin)) evaluated in two patients (2 and 4) showed normal results. Although we excluded a major platelet dysfunction with these tests, aberrant PtdIns(4,5)P_2 and/or RhoGAP–mediated signalling, might, nonetheless, contribute to platelet disorders and CT prolongation.

We first explored by Western blot the OCRL expression in platelets from one LS patient. Indeed, OCRL is ubiquitously expressed but had never been specifically detected in platelets. OCRL was present in the control platelet extract but only a faint band was observed in the platelets from the LS patient (Fig S1).

RhoA is known to play a key role in the early steps of platelet activation, especially shape change and adhesion (Leng *et al*, 1998). To evaluate potential Rho-dependent signalling perturbations in the PFA system, we inhibited ROCK, a downstream target of Rho. Preincubation of blood with Y27632 significantly increased PFA CT for each healthy donor. Median [range] ADP CT was 96 [73–121] s with vehicle and 147 [118–223] s with ROCK inhibitor ($P = 0{\cdot}018$ (Wilcoxon's rank test)), which represented a mean increase of 53% (Fig 1). In contrast, Y27632 had no effect on TRAP-induced platelet aggregation (data not shown). This result is consistent with the discrepant tests of patient's platelet functions, i.e. a prolonged CT with the PFA100 and normal platelet aggregation tests.

The significantly prolonged CT obtained with the ROCK inhibitor has never been shown before. However, Calaminus *et al* (2007) reported the inhibition of stress-fibre formation, under platelet-spreading conditions on collagen-coated coverslips, and markedly reduced colocalization of actin and myosin IIA when platelets were incubated with Y27632. Moreover, platelet aggregation with collagen was unaffected, regardless of whether Y27632 was present or not, which was confirmed by our findings with TRAP as the platelet agonist. Our results further support the good sensitivity of the PFA analyzer to the early steps of platelet activation: adhesion and shape change. This sensitivity might highlight one of the mechanisms involved in the prolonged PFA CT seen in LS. Thus, one can

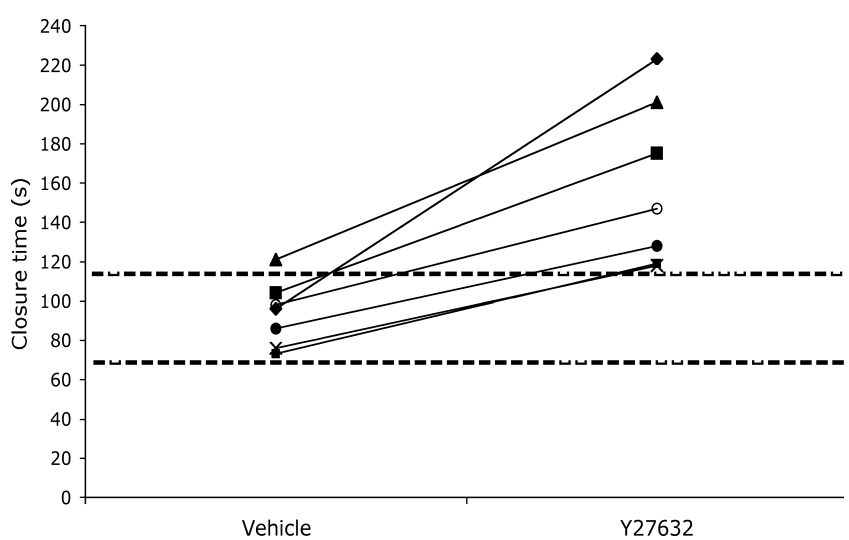

Fig 1. ROCK Inhibitor Effect on ADP Closure Time in the PFA-100 System. Closure times were determined with the CADP (collagen and adenosine 5-diphosphate) cartridge after a 60-s incubation of whole blood samples from seven blood donors with 200 μmol/l Y27632 or vehicle (0·9% NaCl). All samples were tested in duplicate. Horizontal dotted lines indicate the normal range of CADP closure times.

hypothesize that the loss of OCRL perturbs the ROCK activation pathway and through the early steps of platelet activation, but not the platelet aggregation.

Nevertheless, further studies focusing on the implication of the polyinositol 5-phosphatase domain in platelet dysfunction are needed. Indeed, OCRL loss leads to an increase of PtdIns(4,5)P_2 (Zhang *et al*, 1995). PtdIns(4,5)P_2 is implicated in the organization of the actin cytoskeleton (Raucher *et al*, 2000), granule secretion (Flaumenhaft, 2003), microparticule formation (O'Connell *et al*, 2005), procoagulant activity (Bucki *et al*, 1998), and thrombus stability (Lian *et al*, 2005). Moreover, upon platelet activation, the PtdIns(4,5)P_2 concentration is upregulated in membrane raft microdomains, and thus induce the recruitment of several actin-modulating proteins. Therefore, the raft participates in the organization of membrane–cytoskeleton interactions implicated in the regulation of the late phase of platelet activation and clot retraction (Bodin *et al*, 2005). A prospective study is in progress to further investigate the roles of the RhoA pathway and PtdIns(4,5)P_2-level modulations in the haemostatic defect observed in LS.

In conclusion, we have identified a potential causal mechanism involved in platelet-activation anomaly in LS patients. Physicians treating such patients should now be aware of their increased haemorrhagic risk. Therefore, we recommend that LS patients be systematically investigated for haemostasis disorders, particularly before surgery, which is often required in this disease. Moreover, our data contribute to a better knowledge of platelet-signalling pathways.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig S1. Western blot of OCRL from platelets and dermal fibroblasts.

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