Instability of cytosolic phospholipase A₂α variant upon cellular expression as a basis for its clinical presentation

Aida Zulueta¹; Cristina Razzari²; Gessica Fontana³; Eti A. Femía²; Elena M. Faioni²; Marco Cattaneo³; Marco Trinchera³

¹Dipartimento di Scienze della Salute, Università degli Studi di Milano, Milano, Italy; ²Medicina 3, Azienda Ospedaliera San Paolo, and Dipartimento di Scienze della Salute, Università degli Studi di Milano, Milano, Italy; ³Dipartimento di Medicina Clinica e Sperimentale, Università dell’Insubria, Varese, Italy

Dear Sirs,

We recently described two siblings suffering bleeding diathesis and recurrent gastrointestinal ulcers, associated with homozygous 1723G>C transition in PL2G4A gene, which changed Asp575 to His in cytosolic phospholipase A₂α (cPLA₂α) (1). Although recurrent gastrointestinal ulcers were also described in a patient with heterozygous nonsynonymous amino acid substitutions Ser111Pro, Arg485His, and Lys651Arg (2) and in two patients with a homozygous 4 bp deletion (g.155574_77delGTAA) determining a frameshift of 10 amino acids before a premature stop codon (p.Val707fsX10) (3), the bleeding diathesis was a typical feature of our patients. Interestingly, the impairment in arachidonic acid metabolism associated to the Asp575His mutation was exceptionally severe, as shown by the very low levels of serum thromboxane B₂, which were over ten-fold lower than those found in the other cases of inherited cPLA₂α deficiency and in subjects on chronic treatment with aspirin, an irreversible cyclo-oxygenase inhibitor. To characterise the defect further, wild-type (WT) and variant cPLA₂α were permanently expressed in human embryonic kidney HEK-293 cells as fusion proteins with the HaloTag sequence in the pFN21 vector (see Suppl. Methods, available online at www.thrombosis-online.com). Cell transfection and selection were performed following a previously reported procedure (4). Four clones from the WT and 4 from the variant cPLA₂α-transfected HEK-293 cells were selected because of HaloTag expression, as determined by fluorescence microscopy upon vital staining with TMR-ligand. Mean fluorescence intensity was 14.10 ± 8.87 (mean value ± standard deviation) in the WT clones and 3.77 ± 3.25 in the Asp575His clones, respectively, as assessed by flow cytometry (Figure 1a). These values suggest a much lower expression of the mutant construct, although an exception is apparent in the case of clone 4. However, the crude fluorescence intensity could be also affected by the amount of tagged fragments or free tag potentially formed. Expression levels of the HaloTag/cPLA₂α transcript in the clones, quantitated by competitive RT-PCR (see Suppl. Methods, available online at www.thrombosis-online.com), were rather variable, as expected in this expression system, and did not differ between WT and mutant clones (Figure 1b). In particular, mutant clone 1 displayed the highest value measured among all clones, reaching an amount that was just one-half of β-actin transcript. Conversely, the WT HaloTag/cPLA₂α fusion protein was detectable by western blotting with both the anti-HaloTag and the anti-cPLA₂α antibodies as a very prominent band of about 140 kDa in all 4 WT clones, while it appeared as a faint spot in mutant clone 1 only, and was undetectable in the others (Figure 1c). Noteworthy, in mutant clone 4, a spot of much lower molecular weight (close to that of the HaloTag alone) was detected with the anti-HaloTag antibody only, indicating the expression of an aberrant protein lacking the bulk of cPLA₂α sequence (Suppl. Figure 1, available online at www.thrombosis-online.com).

The fusion protein from WT clone 2 and the one from Asp576His clone 1 were both purified through a HaloTag binding resin and eluted by the action of a specific protease (see Suppl. Methods, available online at www.thrombosis-online.com), obtaining the proteins free from the HaloTag peptide. Western blot analysis of the purified proteins showed a good recovery of WT cPLA₂α and a substantial loss of the mutated one (Figure 1d). It is not possible at this stage to establish whether the protein was actually lost or misfolded to such a degree that could not be recognised by the antibody. These results indicate that the WT protein is stable in vitro, while the variant is not. Thus, we were able to reproduce in a cell system the main features reported in the patient’s platelets, where the mRNA transcribed from the mutated cPLA₂α was expressed at the same levels as in other family members, while the protein was undetectable (1). The mutant protein, when detectable, appears only in the cytosol and not in a particulate fraction (see Suppl. Figure 2, available online at www.thrombosis-online.com), suggesting that the mutation does not impair solubility. In conclusion, the 1723G>C transition in PL2G4A gene allows normal mRNA transcription and processing, but gives rise to an unstable protein. This mechanism of disease was already reported for other inherited haematologic (5) and systemic defects (6). According to a recently proposed model (7), the interaction of some molecular chaperones with mutated proteins detects potentially functional but excessively unstable proteins and directs them towards degradation instead of folding. In our expression system, which is able to force expression via both transcription through the strong cytomegalovirus promoter and translation through the HaloTag sequence, we found all mutant clones expressing the fusion transcript, 2 clones not expressing the fusion protein at all, one expressing the HaloTag only, and one expressing a minimal amount of fusion protein, which was lost/misfolded during purification. Since such a clone was the one expressing an enormous amount of transcript, we speculated that subsequent translation saturated the degradation mechanism, providing the minimal amount of fusion-protein

Correspondence to:

Marco Trinchera
Dipartimento di Medicina Clinica e Sperimentale
Università dell’Insubria, Varese, Italy
Tel.: +39 0332 39 7160, Fax: +39 0332 39 7119
E-mail: marco.trinchera@uninsiubria.it

Received: November 10, 2014
Accepted after minor revision: February 24, 2015
Epub ahead of print: April 23, 2015
http://dx.doi.org/10.1160/TH14-11-0926
Thromb Haemost 2015; 114: 208–210

© Schattauer 2015
detected. Preliminary data obtained incubating a mutant clone with the proteasome inhibitor Z-Leu-Leu-Phe indicated a faint but detectable recovery of expression, corroborating such a working hypothesis. Moreover, in the absence of the HaloTag sequence, the native Asp575His cPLA2α appears to be unstable in vitro, per se. Altogether the present data are consistent with a complete absence of active protein under physiological conditions in patients carrying the Asp575His mutation, which is in turn responsible for the severe impairment of arachidonic acid metabolism observed in their platelets.

Acknowledgement
This work was supported in part by a grant from the University of Insubria (FAR2013). A.Z. was supported by the Ph.D. Program in Molecular Medicine of the University of Milan.

Conflicts of interest
None declared.

References

In the original article by Hillis et al. "Acute phase treatment of VTE: Anticoagulation, including non-vitamin K antagonist oral anticoagulants" (Thromb Haemost 2014; 113: 1193-1201) in Table 3 the treatment duration of apixaban should be 7 days not 10 days. The corrected table is printed here.

Thromb Haemost 2015; 114: 210
http://dx.doi.org/10.1160/TH15070002

<table>
<thead>
<tr>
<th>Drug</th>
<th>Dosage – Acute Treatment</th>
<th>Acute Treatment Duration</th>
<th>Half-life (hours)</th>
<th>Renal Clearance</th>
<th>Drug Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parenteral Agents</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unfractionated heparin</td>
<td>Weight-based bolus followed by continuous infusion to maintain therapeutic aPTT (1.5–2 times normal) or 333 U/kg subcutaneously first dose then 250 U/kg q12h*</td>
<td>Monotherapy or minimum 5 days and continued until therapeutic on VKA; edoxaban or dabigatran</td>
<td>1.5</td>
<td>30%</td>
<td>None</td>
</tr>
<tr>
<td>Low-molecular-weight heparins</td>
<td>Monotherapy or minimum 5 days and continued until therapeutic on VKA or TSOAC</td>
<td>3–4</td>
<td>80%</td>
<td>Transferase</td>
<td></td>
</tr>
<tr>
<td>dalteparin</td>
<td>200 IU/kg subcutaneously every 24 h or 100 IU/kg q12h</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>enoxaparin</td>
<td>1.5 mg/kg subcutaneously every 24 h or 1 mg/kg q12h</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>nadroparin</td>
<td>171 IU/kg every 24 h subcutaneously or 86 IU/kg q12h</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tinzaparin</td>
<td>175 IU/kg subcutaneously every 24 h</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Factor Xa inhibitor</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>fondaparinux</td>
<td>5 mg (&lt;50 kg), 7.5 mg (50–100 kg), or 10 mg (&gt;100 kg) subcutaneously every 24 h</td>
<td>minimum 5 days and continued until therapeutic on VKA</td>
<td>17–21</td>
<td>100%</td>
<td>None</td>
</tr>
<tr>
<td>Oral Agents</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Direct factor Xa inhibitors</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rivaroxaban</td>
<td>15 mg bid</td>
<td>3 weeks</td>
<td>7–11</td>
<td>33%</td>
<td>CYP3A4 and P-glycoprotein inducers or inhibitors</td>
</tr>
<tr>
<td>apixaban</td>
<td>10 mg bid</td>
<td>7 days</td>
<td>8–12</td>
<td>25%</td>
<td></td>
</tr>
</tbody>
</table>

* aPTT 6 hours following injection on third day of treatment should be performed to confirmed therapeutic; may be considered for patients at high-risk of bleeding; VKA should be started immediately and parenteral anticoagulant may be discontinued when INR > 2; patients who are pregnant or with active cancer; edoxaban or dabigatran require dual-drug treatment for first 5 days; aPTT, activate partial thromboplastin time; h, hours; VKA, vitamin K antagonist; TSOAC, target-specific oral anticoagulant; CYP3A4, cytochrome P450 3A4.

Erratum to Sala et al. "MiR-143/145 deficiency attenuates the progression of atherosclerosis in Ldlr−/− mice" (Thromb Haemost 2014; 112: 796-802)

In the original article by Sala et al. "MiR-143/145 deficiency attenuates the progression of atherosclerosis in Ldlr−/− mice" (Thromb Haemost 2014; 112: 796-802) the institutional affiliation of Dr. Elia was given incorrectly. The correct author affiliation is IRCCS Multimedica, Milan, Italy.

Thromb Haemost 2015; 114: 210
http://dx.doi.org/10.1160/TH15070001