Scott Syndrome, Characterized by Impaired Transmembrane Migration of Procoagulant Phosphatidylserine and Hemorrhagic Complications, Is an Inherited Disorder

By Florence Toti, Nathalie Satta, Edith Fressinaud, Dominique Meyer, and Jean-Marie Freyssinet

An as yet single family with a bleeding history is shown to present the characteristic lack of membrane expression of procoagulant phospholipids observed in Scott syndrome. Low prothrombin consumption in the serum of the propositus, a 71-year-old woman, and two of her children was the sole abnormal hemostasis parameter. The degree of exposure of procoagulant phospholipids, chiefly phosphatidylserine, was reduced in stimulated platelets, erythrocytes and Epstein-Barr virus-infected B lymphocytes. The data are compatible with homozygous status of the propositus and heterozygous status of her children. Scott syndrome appears to be transmitted as an autosomal recessive trait reflecting the deletion or mutation of a putative outward phosphatidylserine translocase. The detailed knowledge of this transporter could have an impact in membrane physiology.

The completion of the blood coagulation process requires the surface assembly of the characteristic enzyme complexes involving several of the clotting factors. The exposure of negatively charged phospholipids, chiefly phosphatidylserine, sequestered in the inner leaflet of the plasma membrane of resting cells, is a prerequisite for the local concentration of actors playing a fundamental role in the generation of thrombin. Stimulated platelets are thought to be the major source of phosphatidylserine, previously referred to as platelet factor 3. When available at the surface of activated cells or derived fragments, phosphatidylserine acquires a procoagulant character due to its ability to complex vitamin K-dependent factors VII, IX, X, and XI, as well as activated cofactors VIII and V. The surface amplification potential of such a system is linked to the feedback control exerted by thrombin through retroactivation of cofactors VIII and V and platelet stimulation. Vitamin K antagonists enable efficient prevention of thromboembolic disorders by interfering in the maturation process of vitamin K-dependent clotting factors, resulting in an impaired capacity of the latter to associate with procoagulant phospholipids. The symmetrical approach consisting in the pharmacologic modulation of the degree of exposure of phosphatidylserine by stimulated platelets has been hampered by the lack of knowledge of the mechanism governing its transmembrane migration.

The production of a procoagulant response is certainly not the sole consequence of the loss of the asymmetric distribution of phospholipid species between the two leaflets of the plasma membrane, but physiologic implications in cell types other than blood cells remain poorly understood. Ubiquitous cytodynamic events such as endocytosis or exocytosis are accompanied by the loss of membrane asymmetry. Membrane fusion of enveloped viruses with target cells is facilitated in the presence of phosphatidylserine. Programmed cell death, apoptosis, essential in many aspects of normal development and occurring in major diseases such as cancer, acquired immunodeficiency syndrome (AIDS), autoimmune disorders, etc., results in the release of apoptotic fragments bearing exposed phosphatidylserine. Several protective mechanisms could neutralize the pathogenic potential of circulating cell fragments. Phosphatidylserine is a determinant of reticuloendothelial clearance and antiphospholipid antibodies could counteract its procoagulant character. Such antibodies are precisely associated with an increased risk of thrombosis.

The availability of procoagulant phospholipids is essential for a normal hemostatic response, as clearly shown by the severe to moderate bleeding episodes due to a deficiency of platelet procoagulant activity. Several isolated cases were reported, but a unique one has been well documented as Scott syndrome. Scott syndrome seems to be transmitted as an autosomal recessive trait. Scott syndrome was discussed but not confirmed. In a more recent report, Epstein-Barr virus (EBV)-infected B cells from the first case of Scott syndrome were fused with the myeloma cell line UC-LUC, resulting in hybridomas in which the Scott abnormality was corrected. The investigators concluded that it is a genetic disorder affecting the outward transmembrane migration of phosphatidylserine.

PATIENTS AND METHODS

Subject selection. The propositus (Fig 1), a 71-year-old woman born from first cousins, suffered from severe to moderate hemorrhagic episodes for a long time, including epistaxis, trauma-related hematomas, and at childbirths. She has been successfully treated using whole blood transfusions. Her older sisters (II-1 and II-31 died from hemorrhage in childbirth. Her children (III-6 to III-10), nephews (III-1 and III-3 to III-5), and grandchildren (IV-1 to IV-20) denied any bleeding tendency. No

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other recurrent symptom was reported by any of the members of this family. The nature of the study was explained to the patient, one of her sons [III-8], two of her daughters [III-9 and III-10], two of her grandnieces [IV-1 and IV-2] (nephew [III-1]'s daughters), and 10 control subjects who gave fully informed consent.

**Hemostasis parameters, cell preparations, and culture.** The hemostasis parameters were determined according to standard routine procedures. For studies performed with cells, blood samples from the propositus, her son [III-8], and her daughter [III-10] were collected in National Institute of Health (NIH) formula A acid-citrate-dextrose (1 part anticoagulant:6 parts blood) and immediately processed for platelet and red blood cell isolation. Lymphocytes were isolated under sterile conditions and stored in liquid nitrogen. Infection of B lymphocytes by EBV (EBV B958, Marmouset) was performed in RPMI culture medium containing 20% fetal calf serum (FCS; Life Technologies Inc, Paisley, UK) in the presence of 50 ng/mL cyclosporine and was achieved within 3 weeks. EBV-infected B cells were expanded in standard RPMI culture medium supplemented with 5 mmol/L HEPES, 5% FCS, 5% Hyclone serum (Hyclone Laboratories Inc, Logan, UT), and 50 ng/mL gentamicin. In some experiments, cells were cultured in the presence of recombinant human interleukin-4 (IL-4; 1 ng/mL; Promega Corp, Madison, WI) to reduce mortality. Cell mortality was estimated by Trypan blue staining (0.1% final concentration).

**Functional detection of procoagulant phospholipids.** Procoagulant phospholipid exposure in stimulated cells and derived microparticles was detected using a human prothrombinase assay in which phosphatidylserine promotes the activation of prothrombin by factor Xa in the presence of factor Va. Thrombin generated by assembled prothrombinase complex was measured using a chromogenic assay as described elsewhere. Red blood cells, platelets, and EBV-infected lymphocytes were studied at 10^7 cells/mL, 7 x 10^7 cells/mL, and 2 x 10^4 cells/mL, respectively. The ability of all cell type to expose procoagulant phosphatidylserine was examined after stimulation by 0.1 NIH U/mL human α-thrombin, 12 μg/mL equine tendon collagen (Hormon-Chemie, Munich, Germany) or 5 μmol/L calcium ionophore A23187 for 10 minutes at 37°C for platelets, 5 μmol/L A23187 for 90 minutes at 37°C for red blood cells, 5 μmol/L A23187 for 10 minutes at 37°C for EBV-infected B cells, in the presence of 2 mmol/L external CaCl_2 for each cell type. Red blood cells and EBV-infected B lymphocytes were separated from derived microparticles by centrifugation at 12,000g for 30 seconds before the measurements. In each case, results were compared with the prothrombinase activities developed in samples from healthy volunteers. To assess whether the phosphatidylserine content of any cell type was normal, cells were disrupted by three successive freezing/thawing/sonication steps, and the resulting cell fragment suspensions were used in the prothrombinase assay as described above.

**Flow cytometry.** Analysis of the platelet membrane status was performed using annexin V as a protein probe of phosphatidylserine exposure. Annexin V was purified from human placenta and conjugated to fluorescein isothiocyanate (FITC), as already described. Ionophore-stimulated (see above) and nonstimulated cell suspensions were incubated with 5 μg/mL annexin V<sup>FITC</sup> for 10 minutes at room temperature in the presence of 0.5 mmol/L calcium before data acquisition using a Becton Dickinson flow cytometer (Becton Dickinson, San Jose, CA). Parameters were set to assess phosphatidylserine exposure evidenced by annexin V<sup>FITC</sup> binding and recorded on FL1 fluorescence scale, simultaneously for cells and derived microparticles that were distinguished according to size (forward light scatter). For each sample, 10,000 events were recorded and further analyzed in the appropriate gate delineated on dot plots, as previously described.

Analysis of the ability of red blood cells to undergo shape change and membrane vesiculation after stimulation by ionophore (see above) was performed using forward-angle versus right-angle light scatter dot plot representation of control samples, reflecting minimal availability of negatively charged phospholipids for prothrombinase complex assembly in each case. Exposure of procoagulant phospholipids remained weak after various stimulations of propositus’ cells when compared with samples from healthy subjects (Figs 2 and 3). Prothrombinase activities in stimulated propositus’ red blood cells and corre-
Platelet suspensions were either unstimulated (UnStd) platelets from the propositus [II-5] measured by prothrombinase activity or stimulated by the association of thrombin and collagen (Thr/Coll; 0.1 NIH UlmL) for 10 minutes at 37°C. Stimulations and measurements were performed in the presence of 2 and 1 mmol/L external CaCl₂, respectively. Data are expressed as the mean ± SEM; n = 3 for the platelets from the propositus and her offspring, and n = 10 for platelets from control subjects.

The defect of phosphatidylserine exposure was more easily evidenced in red blood cells that elicit a delayed response to calcium ionophore. Erythrocytes from the propositus were almost insensitive to ionophore treatment. Stimulated red blood cells from the daughter exhibited about twice as much prothrombinase activity as those of her mother, whereas the son's cells produced a response of about 60% of normal (Fig 3). Diminished prothrombinase activity was also observed in supernatants of corresponding stimulated red blood cells from the three members of the family, testifying to a reduced ability to shed procoagulant microparticles. Again, the propositus' cells showed the most pronounced defect.

Procoagulant phospholipid exposure occurred at comparable basal levels in nonstimulated control and patient's EBV-infected B lymphocytes (Fig 4), probably as a result of cell mortality, which was estimated between 10% and 15% in both populations, depending on the culture stage. Mortality could be reduced by about 30%, with concomitant reduction of the same order of basal prothrombinase activity, when cells were cultured in the presence of IL-4 (data not shown). Surprisingly, the mortality of offspring's nonstimulated EBV-B cells was consistently lower, ranging between 3% and 8%, resulting in lower basal prothrombinase activity. Ionophore treatment of control EBV-B cells was responsible for the development of a substantial phospholipid-dependent procoagulant activity almost equally distributed between cells and microparticles. The overall prothrombinase activity (cell and microparticle-associated) of the stimulated procoagulant phospholipid externalization in stimulated platelets from the propositus [II-5] measured by prothrombinase assay. Platelet suspensions were either unstimulated (UnStd) or stimulated by the association of thrombin and collagen (Thr/Coll; 0.1 NIH U/ml and 0.12 μg/ml final respective concentrations) for 10 minutes at 37°C or by the calcium ionophore A23187 (A23187; 5 μmol/L final concentration) for 10 minutes at 37°C. Stimulations and prothrombinase activity measurements were performed in the presence of 2 and 1 mmol/L external CaCl₂, respectively. Data are expressed as the mean ± SEM; n = 3 for the platelets from the propositus and her offspring, and n = 10 for platelets from control subjects.

**Table 1. Assessment of the Hemostasis Parameters in the Propositus [II-5], Her Son [III-8], and Her Daughter [III-10]**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Propositus II-5</th>
<th>Son III-8</th>
<th>Daughter III-10</th>
<th>Normal Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bleeding time (min)</td>
<td>3.30</td>
<td>ND</td>
<td>ND</td>
<td>2.30-9.30</td>
</tr>
<tr>
<td>Platelet count (giga/L)</td>
<td>395</td>
<td>223</td>
<td>237</td>
<td>150-500</td>
</tr>
<tr>
<td>Prothrombin time (s)</td>
<td>12.7</td>
<td>11.9</td>
<td>11.9</td>
<td>12-14</td>
</tr>
<tr>
<td>aPTT (s)</td>
<td>38</td>
<td>34</td>
<td>35</td>
<td>33-38</td>
</tr>
<tr>
<td>Serum prothrombin time (s)</td>
<td>11.8</td>
<td>15.2</td>
<td>15.7</td>
<td>&gt;25</td>
</tr>
<tr>
<td>Residual serum prothrombin (%)</td>
<td>68</td>
<td>5.5</td>
<td>7.5</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Coagulation factors</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FVIII (%)</td>
<td>105</td>
<td>102</td>
<td>72</td>
<td>50-200</td>
</tr>
<tr>
<td>FV (%)</td>
<td>&gt;120</td>
<td>ND</td>
<td>ND</td>
<td>80-120</td>
</tr>
<tr>
<td>FIX (%)</td>
<td>94</td>
<td>ND</td>
<td>ND</td>
<td>70-120</td>
</tr>
<tr>
<td>FIXII (%)</td>
<td>&gt;120</td>
<td>ND</td>
<td>ND</td>
<td>80-120</td>
</tr>
<tr>
<td>vWF (ristocetin cofactor) (%)</td>
<td>118</td>
<td>90</td>
<td>75</td>
<td>50-200</td>
</tr>
<tr>
<td>Fibrinogen (g/L)</td>
<td>3.5</td>
<td>3</td>
<td>2.86</td>
<td>2.4</td>
</tr>
</tbody>
</table>

Abbreviation: ND, not determined.
The propositus and her offspring, and respectively, lute values were similar. Phosphatidylserine and microparticle shedding by the patient's platelets compared with control platelets exceedingly stimulated by ionophore in control cells, showing that the absolute values were similar.

Disrupted platelets, red blood cells, and EBV-infected B cells from the propositus and her children yielded the same prothrombinase activity as that of corresponding control cells treated likewise, showing a normal content of procoagulant phospholipids.

Flow cytometry analysis using annexin VFITC allowed us to gather evidence regarding the defective exposure of phosphatidylserine and microparticle shedding by the patient's platelets compared with control platelets exceedingly stimulated by calcium ionophore (Fig 5). The latter underwent massive phosphatidylserine exposure and membrane vesiculation. The patterns corresponding to the binding of annexin VFITC to stimulated cells and derived microparticles were in accordance with prothrombinase activity measurements (Fig 2).

Red blood cells from the patient appeared refractory to shape change and showed reduced vesiculation after treatment with 5 μmol/L calcium ionophore for 90 minutes at 37°C in the presence of 2 mmol/L external Ca²⁺, whereas her offspring’s cells elicited an intermediate response (Fig 6). The dot plot representation of forward-angle versus right-angle light scatter enabled us to clearly distinguish two populations. Cells showed the highest forward and side scatter signals, whereas derived microparticles had lower ones. Less than 1.5% of total events (cells + debris and/or machine noise) was detected in the microparticle gate in any of untreated cell types. With stimulated red blood cells, the proportion of microparticles was, respectively, 19.5%, 5%, 10%, and 8% for the normal subject’s, propositus’, daughter’s, and son’s cells.

No difference was noticed in the electrophoretic patterns of cytoskeletal proteins of patient’s and control platelets (data not shown).

**DISCUSSION**

The above results clearly establish close links between the almost total absence of phosphatidylserine exposure by in vitro stimulated platelets from the propositus and her hemorrhagic diathesis. The defect was observed not only with the physiologically relevant agonist combination of thrombin and collagen, but also under more drastic conditions using calcium ionophore. The latter was not able to induce significant phosphatidylserine externalization in patient’s red blood cells when compared with corresponding control cells. The reduction of procoagulant phospholipid exposure was also highly significant in her EBV-infected B cells despite interference of cell mortality. Two of her children could be explored for the same anomaly. The overall phospholipid-dependent procoagulant response did not exceed 30% to 55% of normal with the daughter’s cells, whereas the son’s cells showed a behavior closer to normal but consistently lower. These observations are in agreement with measurements of residual prothrombin in serum and have been confirmed by flow cytometry. It has to be emphasized that residual serum prothrombin is a complex function of the degree of availability of procoagulant lipids in whole blood. This could explain the differences of serum prothrombin time and residual serum prothrombin between the propositus and her children. The absence of bleeding in the offspring might precisely be related to residual serum prothrombin close to normal. In our routine practice, under no circumstance of unexplained hemorrhagic disorder, these parameters were found to be abnormal except in this family. The defect of phosphatidylserine exposure and consecutive reduced membrane vesiculation was further evidenced by the reduced binding of a fluorescent derivative of annexin V used to detect available phosphatidylserine. It has to be pointed out that none of the above defects could be due to abnormal composition of membrane phospholipids because patient’s cell fragments bearing fully scrambled phospholipids produced a phosphatidylserine-dependent procoagulant response identical to that of control cells when lysed likewise.

In a first instance, these observations appear compatible with homozygous status of the propositus while her children could be heterozygous with respect to Scott syndrome, which would be transmitted as an autosomal recessive trait. The patient’s sisters can reasonably be considered as homozygous because they died from documented hemorrhage. The
possibility of compound heterozygous status appears improbable because it should have been transmitted to the three sisters. However, it has to be emphasized that such conclusions should be viewed as only partly qualified because the genetic analysis could not be performed due to the absence of candidate gene(s). In subjects born from first cousins, $\frac{1}{8}$ of the genome has a homozygous character that implies that at least six or seven subjects of this type have to be examined for localization of mutated gene or regulatory element(s) by mapping homozygous loci. Unfortunately, such a study could not be performed here for obvious reasons. The first experimental evidence that a gene defect can give rise to Scott syndrome was reported recently. Our familial study confirms that it is indeed a genetic disorder. The identification of candidate gene(s) is now the next goal but remains dependent on the feasibility of functional rescue methodology in Scott EBV-B cell lines.

The maintenance of the asymmetric distribution of phospholipid species between the two leaflets of the plasma membrane of a variety of cells involves a specific inward transporter of aminophospholipids, chiefly phosphatidylethanolamine and phosphatidylserine. Although the gene coding for this transporter remains to be identified, it is tempting to speculate that aminophospholipid translocase activity is increased in Scott syndrome. This would explain the sustained sequestration of phosphatidylserine in the inner membrane leaflet. However, experiments performed by other investigators who had access to the red blood cells from the above Scott subjects excluded this possibility as the inward phosphatidylserine transport was found normal. In addition, once incorporated by red blood cells from the propositus, labeled phosphatidylserine could not be re-externalized (A. Zachowski and J.-C. Sulpice, personal communication, February 28, 1995). Identical preliminary observations were also reported by another group who had access to the cells of the first case of Scott syndrome.

The flip-flop or transmembrane migration of phospholipids remains mostly mysterious. Nevertheless, a phosphatidylcholine translocase has been recently characterized using a gene disruption approach. It is the product of the $mdr2$ gene belonging to the family of P-glycoprotein multidrug transporters. Its absence leads to liver disease due to the lack of phosphatidylcholine in bile. Because aminophospholipid translocase activity appears normal in Scott red blood cells, it seems reasonable to hypothesize that the Scott defect could be due to a mutation affecting a putative outward phosphatidylserine or aminophospholipid translocase. Another possibility would be that aminophospholipid translocase operates as both an inward and outward transporter, because several pumps do, depending on particular regulation. In this case, only the outward function would be impaired. This hypothesis could be explored using cell mutants defective in inward phosphatidylserine transport recently described.

A more general perspective of the identification of the gene(s) and corresponding product(s) involved in phosphatidylserine or amino-phospholipid externalization is to assess whether it is a more ubiquitous feature involved in the processes regulating communication between cells and their environment under various physiologic or pathologic circumstances. In this respect, Scott syndrome would be an additional example of the prime...
usefulness of genetic variants for the characterization of as yet unknown physiologic mechanisms.

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