Increased Expression of Phospholipid Scramblase 1 in Monocytes from Patients with Systemic Lupus Erythematosus

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ABSTRACT. ***Objective.*** A high incidence of thromboembolic events has been reported in patients with systemic lupus erythematosus (SLE). Phosphatidylserine (PS) is normally sequestered in the inner leaflet of cell membranes. Externalization of PS during cell activation is mediated by phospholipid scramblase 1 (PLSCR1) and has a central role in promoting blood coagulation. We investigated the underlying pathogenic status of thrombophilia in SLE by analyzing PLSCR1 expression on monocytes from patients with SLE.

Methods. Sixty patients with SLE were evaluated. Twenty-three patients had antiphospholipid syndrome (APS/SLE). Plasma D-dimer levels were measured as a marker of fibrin turnover. The cDNA encoding human PLSCR1 was cloned from the total RNA extract from monocytes, and independent clones were sequenced. PLSCR1 mRNA expression in CD14+ cells was determined by real-time polymerase chain reaction. PS exposure on CD14+ cell surface was analyzed by flow cytometry.

Results. Elevated D-dimer levels were found in plasma from SLE patients. Three splice variants of PLSCR1 mRNA were identified in all subjects, and levels of full-length PLSCR1 mRNA were significantly increased in SLE compared to healthy controls (2.9 ± 1.5 vs 1.3 ± 0.4, respectively; $p < 0.0001$). Flow-cytometry analysis showed relative enhancement of PS exposure in the surface of CD14+ cells in SLE patients compared to healthy controls.

Conclusion. Novel PLSCR1 splice variants were identified. Monocytes in SLE patients had enhanced PLSCR1 mRNA expression, as well as increased fibrin turnover and cell-surface PS exposure, indicating that PLSCR1 may, in part, contribute to the prothrombotic tendency in SLE. (First Release June 1 2010; J Rheumatol 2010;37:1639–45; doi:10.3899/jrheum.091420)

Key Indexing Terms:
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Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by acute/chronic inflammation linked with the production of autoantibodies, generation of circulating immune complexes, and activation of the complement system. Thrombotic events are frequent manifestations observed in more than 10% of patients with SLE[1,2]. The etiology of thrombosis is multifactorial and might be related to premature atherosclerosis, vasculitis, and hypercoagulability. Several environmental or genetic risk factors have been reported to increase this tendency, but the precise thrombotic mechanisms in SLE are not yet clarified[3].

Hypercoagulability in SLE is typically due to the presence of antiphophospholipid antibodies (aPL), complicated with the antiphospholipid syndrome (APS)[4]. aPL belong to a large family of autoantibodies directed against phospholipid-binding plasma proteins or against the complex of these proteins with anionic phospholipids[5].

Phosphatidylserine (PS) is an anionic phospholipid normally sequestered in the inner leaflet of the cell membrane. Externalization of PS occurs in activated cells and plays a

central role in promoting blood coagulation, as PS serves as a catalytic surface for the assembly of the coagulation factors, including the prothrombinase and tenase complex[6]. The exposure of PS at the outer leaflet of the plasma membrane is also essential for the binding of aPL to procoagulant cells. Several groups have reported the crucial role of the p38 mitogen-activated protein kinase (MAPK) pathway in aPL-mediated cell activation[7,8]. In order that aPL bind and activate cells, the immune complexes have to be present on the PS-exposed cell surface[9]. One of the key molecules involved in regulation of PS externalization during cell activation is phospholipid scramblase 1 (PLSCR1), which catalyzes rapid transbilayer movement of phospholipids between membrane leaflets. PLSCR1 is a lipid-raft associated type II plasma protein of about 37 kDa containing 318 amino acid residues with a long N-terminal cytoplasmic domain, a transmembrane helix region, and a short extracellular tail[10].

Although plasma membrane asymmetry is the rule for normal cells, loss of asymmetry, especially the appearance of PS at the cell surface, is associated with physiological and pathological phenomena, including thrombosis[11]. In order to investigate the underlying pathogenic status of thrombophilia in SLE, we examined the expression of PLSCR1 on procoagulant cells from patients with SLE.

MATERIALS AND METHODS

Patients. Sixty unselected consecutive Japanese patients with SLE who visited the Rheumatic and Connective Tissue Disease Clinic were recruited. All patients, 57 women and 3 men, mean age 45 years (range 23–70 yrs), fulfilled the American College of Rheumatology criteria for SLE[12]. Twenty-three patients (38%) were diagnosed as having APS in association with SLE. APS was diagnosed according to the revised international criteria for APS[13].

The mean SLE disease duration was 15.5 ± 9.9 years (range 0.5–42 yrs). No patient had thrombosis or pregnancy complications within 3 months before blood collection. Signs of acute thrombosis were not detected in any patient at the time blood was drawn. Lupus activity was assessed by certified rheumatologists at the time of blood sampling by analyzing laboratory data such as white blood cells and platelet count, hemoglobin levels, C-reactive protein (CRP), C3, C4 and CH50 levels, anti-DNA antibodies, and clinical manifestations. The historical profile of clinical and laboratory manifestations and SLE Disease Activity Index (SLEDAI)[14] were verified by the authors using medical records, as summarized in Table 1.

When blood was drawn, 8 patients were receiving warfarin (13%). No patients were taking heparin.

Blood samples were also collected from 43 apparently healthy Japanese individuals who consented to join the study (25 women and 18 men, mean age 28 yrs, range 20–38 yrs).

The study was performed in accordance with the Declaration of Helsinki and the Principles of Good Clinical Practice. Approval was obtained from the local ethics committee, and informed consent was obtained from each subject before enrollment.

Plasma samples. Venous blood was collected into tubes containing sodium citrate and was centrifuged immediately at 4°C. Plasma samples were depleted of platelets by filtration then stored at –80°C.

Plasma D-dimer determination. Plasma D-dimer levels (Nanopia, D-dimer, Daiichi Kagaku, Tokyo, Japan) were measured as markers of fibrin turnover. The cutoff level was previously defined as > 95th percentile of 65 healthy subjects (34 women. 31 men, mean age 40 yrs, range 18–76), as a routine laboratory assay.

Table 1. Profile of patients.

Characteristic		
Diagnosis, no. (%)		
Non-APS	37	(62)
APS	23	(38)
Age, yrs, mean (range)	45	(23–70)
Female:male	57:3	
Historical manifestations, no. (%)		
Photosensitivity	26	(43)
Oral ulcers	16	(27)
Skin	37	(62)
Arthritis	42	(70)
Serositis	14	(23)
Nephritis	30	(50)
Central nervous system	18	(30)
Hematological	50	(83)
Thrombosis	23	(38)
Arterial	14	(23)
Venous	12	(20)
Arterial and venous	2	(3)
Pregnancy morbidity	6	(11)
At the time of blood testing*		
SLEDAI, mean (range)	2.5	(0–10)
aPL		
aCL IgG/M	8/2	(13)/(3)
Anti-ß$_2$-GPI IgG/M	11/3	(18)/(5)
LAC	20	(33)
Other laboratory data, mean ± SD (range)		
White blood cells, × $10^3/\mu$l	6.8 ± 2.7	(2.1–14.1)
Platelets, × $10^3/\mu$l	230 ± 75	(88–433)
Hemoglobin, g/l	12.5 ± 1.7	(8.2–16.9)
CRP, mg/dl*	0.09	(0.02–3)
C3, mg/dl	85.3 ± 22.1	(46–148)
C4, mg/dl	18.6 ± 8.1	(4–41)
CH50, U/ml	45.3 ± 12.2	(17.3–72)
Anti-DNA antibodies, IU/ml*	5	(0–59)

* Median (range). SLE: systemic lupus erythematosus, APS: antiphospholipid syndrome, aPL: antiphospholipid antibodies, aCL: anticardiolipin antibodies, anti-ß$_2$-GPI: anti-ß$_2$-glycoprotein I antibodies, CRP: C-reactive protein, IU: international units.

Antiphospholipid antibody determination. IgG and IgM anticardiolipin antibodies (aCL) were assayed according to the standard aCL enzyme-linked immunosorbent assay (ELISA)[15]. IgG and IgM anti-ß$_2$-glycoprotein I (anti-ß$_2$-GPI) antibodies were determined by in-house ELISA as described[16]. Normal ranges of IgG (< 18 GPL) and IgM (< 30 MPL) aCL, and IgG (< 2.2 U/ml) and IgM (< 6.0 U/ml) anti-ß$_2$-GPI antibodies, were established using 132 healthy controls with 99th percentile cutoff values.

For the detection of lupus anticoagulant (LAC) guidelines recommended by the Subcommittee on Lupus Anticoagulant/Antiphospholipid Antibody of the Scientific and Standardisation Committee of the International Society of Thrombosis and Haemostasis were followed[17].

Isolation and preparation of cells. Venous blood was collected into tubes containing heparin and processed at room temperature (RT) within 3 hours. Peripheral blood mononuclear cells (PBMC) were isolated on Ficoll-Paque plus® gradient centrifugation (Amersham Biosciences, Uppsala, Sweden) using standard protocols. PBMC were pelleted by centrifugation, and washed twice with phosphate buffered saline (PBS; Sigma, St Louis, MO, USA). Contaminated red blood cells were then lysated with red blood cell

lysis buffer (eBioscience, San Diego, CA, USA) at RT for 10 min and washed twice with PBS. Monocytes were purified using CD14 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) as follows: PBMC pellet was suspended in 80 μl of auto MACS^{TM} rinsing solution (Miltenyi Biotec), and 20 μl of CD14 microbeads were added. After 15 min incubation at 4°C, cells were washed with 2 ml of MACS rinsing solution, suspended in 500 μl of MACS rinsing solution, and separated in a magnetic separation kit (Miltenyi Biotec) according to manufacturer's instructions.

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR). Total RNA were isolated from PBMC or monocytes using RNeasy Mini Kit (Qiagen, Valencia, CA, USA) and reverse-transcribed with the SuperScript^{TM} First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA). cDNA were amplified using a pair of primers corresponding to sequences residing at the beginning and the end of the coding domain of the normal human PLSCR1 transcript noted at GenBank accession NM_021105. The gene-specific primer sequences were as follows: forward 5'-GCT CTC TGG ACC TTG TCT CG-3' and reverse primer 5'-CCA GAG CTA CAG GCC TTA CAG-3'. PCR was performed in 31 cycles of 95°C for 30 s, 61°C for 30 s, and 72°C for 45 s, followed by a final extension step 72°C for 7 min. The amplified products were resolved in 9% polyacrylamide gels, stained with ethidium bromide, and visualized under ultraviolet light. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as control using the following gene-specific primer sequences: forward 5'-ACA TCG CTC AGA CAC CAT GG-3' and reverse 5'-GTA GTT GAG GTC AAT GAA GGG-3'. RT-PCR for GAPDH was performed in 28 cycles of 95°C for 45 s, 54°C for 45 s, and 72°C for 45 s, followed by a final extension step at 72°C for 7 min. Bands of 150 bp were identified for GAPDH in 9% polyacrylamide gels.

Cloning and sequencing of PLSCR1 cDNA. Amplification of PLSCR1 was done using the PCR method described above. PCR products were separated by electrophoresis in 1% agarose gels and visualized with ethidium bromide. Four fragments of 1122 bp, 905 bp, 750 bp, and 551 bp were identified, separately recovered from the gel using MiniElute Gel Extraction Kit (Qiagen), and subsequently cloned into a pcDNA3.1 V5-His©-TOPO® TA expression kit (Invitrogen). Transformed *Escherichia coli* clones were randomly selected and screened with the QIAprep Spin Miniprep kit (Qiagen) and BstX1 restriction enzyme (New England BioLabs, Beverly, MA, USA). Nucleotide sequences of independent clones from samples of 2 healthy donors were determined using a Centri Spin^{-20} column (Princeton Separations, Adelphi, NJ, USA), and ABI Prism 3130 Genetic Analyzer (Applied Biosystems, ABI, Foster City, CA, USA). Sequence alignments were analyzed by the AlignIR version 1.2 (LI-COR) software system, and homology search was carried out using BLAST programs.

Quantitative real–time PCR. Quantitative analysis of gene expression was performed by real-time PCR using ABI Prism 7700® Sequence Detection System (Applied Biosystems) and gene-specific TaqMan MGB probe for PLSCR1 (Hs01062169_m1) (Applied Biosystems), which recognize the junction sequence between exon 3 and 4 of human PLSCR1. The level of the PLSCR1 transcript was normalized to that of the GAPDH using TaqMan MGB GAPDH probe (Hs99999905_m1). Relative quantification was done using the comparable cycle threshold method as described[18].

Western blot analysis. For Western blot analysis of PLSCR1 protein, PBMC from a healthy donor were incubated in the presence or absence of interferon-α2a (IFN, 400 IU/ml; Santa Cruz Chemical Co., Santa Cruz, CA, USA) for 24 h in a 5% CO_2 atmosphere at 37°C. Cells were kept in RPMI-1640 medium (Sigma) supplemented with 10% fetal calf serum (Gibco BRL, Paisley, UK) containing penicillin and streptomycin. After incubation, PBMC were lysed in 30 μl × 10^6 cells of lysis buffer (2% NP-40 in PBS containing 5 mM EDTA, 50 mM benzamidine, 50 mM N-ethylmaleimide, 1 mM phenylmethylsulfonyl fluoride, and 1 mM leupeptin) at 4°C for 1 h. The cell lysates were centrifuged and the supernatants were collected. Concentration of protein in the supernatants was determined using bicinchoninic acid (BCA) assay (Thermo, Rockford, IL, USA).

Supernatants were denatured at 100°C for 5 min in 10% sodium dodecyl sulfate (SDS) sample buffer with 2% ß-mercaptoethanol, subjected to electrophoresis in SDS-PAGE gels (20 μg protein/lane), and transferred to nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). The membrane was cut into 3 pieces and blocked with PBS containing 4% low-fat milk (Yukijirushi, Co. Ltd., Hokkaido, Japan) for 1 h at RT. After 2 washes with PBS-Tween 0.05%, each membrane was probed overnight at 4°C, with one of the following monoclonal antibodies diluted in 1% BSA-PBS: (1) mouse monoclonal anti-human antibody (1E9) to scramblase 1 (abcam) (1.5 μg/ml); (2) mouse monoclonal anti-human antibody (1.5 μg/ml; Life Span Biosciences, Inc., Seattle, WA, USA); or (3) PLSCR1 monoclonal antibody (M12), clone 1E11, 1.0 μg/ml (Abnova, Taipei, Taiwan). After 2 washes, membranes were exposed to horseradish peroxidase-conjugated goat anti-mouse IgG antibody (1:5000; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) in 1% BSA-PBS at RT for 1 h. Immunoreactive proteins were visualized using enhanced chemiluminescence assay (Amersham Biosciences, Piscataway, NJ, USA) and the optical imaging system (Multi Gauge ver. 3.0, LAS-4000 min, Fujifilm, Japan).

Measurement of cell-surface PS exposure. Cell-surface exposure of PS was evaluated by flow-cytometry using FACS Calibur (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) with the Cell Quest program. Monocytes were isolated, as described above, from the subjects who agreed to the double blood collection. Monocytes from 29 patients and 24 healthy subjects had double staining with Annexin-V-Fluos labeling kit (Roche Applied Science, Penzberg, Germany) and BD Pharmingen^{TM} PE anti-human CD14 antibody (BD Bioscience Pharmingen, Franklin Lakes, NJ, USA) for 15 min at 4°C and exposed to FACS analysis. From each sample, data from 10,000 counted-gated viable cells were collected and data were expressed as the percentage of gated CD14+ cells, annexin V+ cells in the total gated cell population.

Statistical analysis. Statistical evaluation was performed by Student's t-test. Either Spearman's rank correlation coefficient or Pearson's correlation were used for analyzing the correlations as appropriate. The significance level was set at $p < 0.05$.

RESULTS

Plasma D-dimer levels. Thirty-three out of 60 SLE patients (55%) had elevated plasma D-dimer. Levels of D-dimer were significantly higher in SLE as compared with those in healthy subjects (1.3 ± 0.7 vs 0.6 ± 0.2 g/ml, respectively; $p < 0.0001$). There was no difference in plasma D-dimer levels between patients with APS and those without.

Antiphospholipid antibodies. aCL and anti-ß$_2$-GPI antibodies were found positive in 17% and 22% of the patients, respectively. Isotype distribution is shown in Table 1. Titers of aCL in positive samples ranged from 18.5 to 80 GPL for the IgG isotype, and 51 to 54 MPL for the IgM isotype. Among patients with positive anti-ß$_2$-GPI antibodies, titers ranged from 2.2 to 105 U/ml for the IgG isotype, and 8.4 to 40 U/ml for the IgM isotype. LAC was positive in 33% of the patients.

Expression of PLSCR1 mRNA in monocytes. RT-PCR was performed to amplify the full-coding sequence of PLSCR1 mRNA. Polyacrylamide gel showed that the PCR product of monocytes had 4 bands, of 1122 bp, 905 bp, 750 bp, and 551 bp (Figure 1). To compare the expression pattern of PLSCR1, total RNA samples from monocytes of 25 SLE patients (15 with APS) and 21 healthy controls were exam-

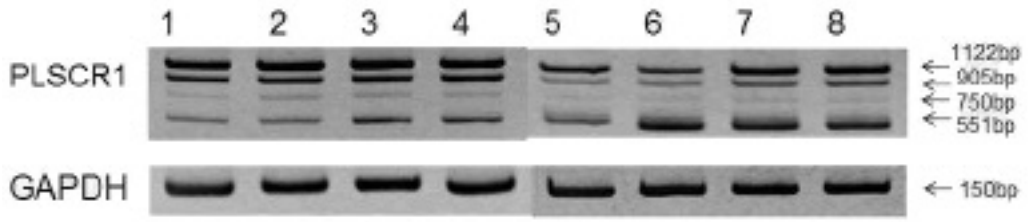

Figure 1. PLSCR1 RT-PCR in CD14+ cells from healthy controls and patients with systemic lupus erythematosus (SLE). RT-PCR products are resolved in 9% polyacrylamide gel. Bands correspond to PLSCR1 and GAPDH products of total RNA from 4 healthy individuals (lanes 1-4), 2 patients with SLE and antiphospholipid syndrome (APS) (lanes 5 and 6), and 2 patients with SLE without APS (lanes 7 and 8).

ined. The intensities of each splice variant differed among the individuals, but there was no specific pattern in the splice variant band intensity in patients with SLE. Because the variants did not turn to the protein, the biological significance of splice variants was not specified.

Sequencing analysis of PLSCR1 splice variants. One fragment of 1122 bp and a mixture of 905 bp, 750 bp, and 551 bp fragments were inserted into the pcDNA3.1 vector. Eighteen independent transformed cell clones were identified to have the expected inserts. DNA sequencing of these clones showed that 6 contained the 1122 bp DNA fragment corresponding to the complete coding sequence for PLSCR1 (GenBank accession NM_021105). Seven clones contained the 905 bp fragment and were identical to the PLSCR1 cDNA, except for a deletion of a 218 bp fragment corresponding to full exon 4 of PLSCR1 as revealed by the LI-COR AlignIR software. Three clones contained the 750 bp fragment and were identical to the PLSCR1 cDNA, except for the deletion of 2 fragments of 218 bp and 221 bp, which correspond to full exons 4 and 6 of PLSCR1, respectively. Two clones contained the 551 bp fragment and were identical to the PLSCR1 cDNA except for the deletion of 3 fragments of 218 bp, 221 bp, and 162 bp fragments corresponding to full exons 4, 6, and 7 of PLSCR1, respectively. Sequence homology searches with BLAST programs revealed that those sequences might be alternative splice variants of human PLSCR1 mRNA (Figure 2).

Western blotting for PLSCR1. Cell lysate products from IFN-activated PBMC of one healthy donor were assayed by Western blotting using 3 monoclonal antibodies directed to 3 different epitopes of PLSCR1. A single band at 37 kDa corresponding to full-length PLSCR1 was detected in IFN-activated cells (Figure 3).

Evaluation of full-length PLSCR1 mRNA levels by real-time PCR. Mean levels of full-length PLSCR1 mRNA were significantly higher in SLE patients than in controls (2.9 ± 1.5 vs 1.3 ± 0.4, respectively; $p < 0.00001$). PLSCR1 mRNA distribution is shown in Figure 4. No statistically significant correlations were found between levels of PLSCR1 mRNA expression and the titers of anti-DNA antibodies, IgG/M aCL, or IgG/M anti-$ß_2$-GPI antibodies. SLEDAI and CRP levels did not correlate significantly with levels of PLSCR1 expression.

Cell-surface PS expression on CD14+ cells. Flow-cytometric analysis showed that the amount of expressed PS on cell

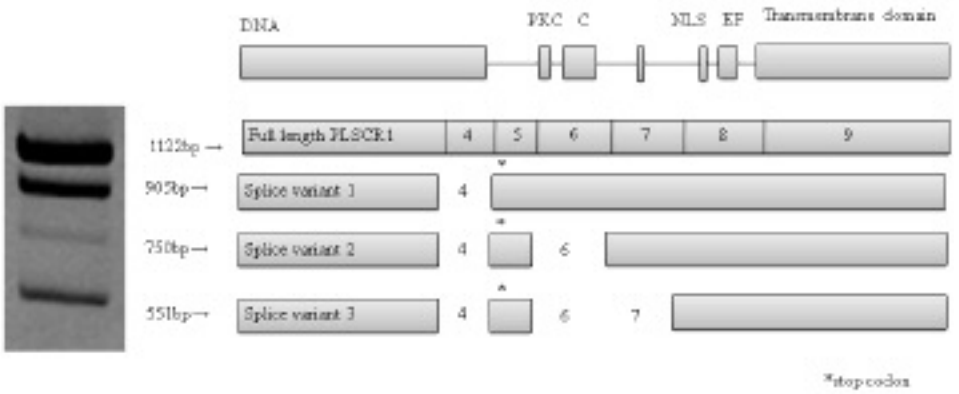

Figure 2. Human PLSCR1 mRNA isoforms. Top panel shows the exon structure and corresponding functional motifs of human PLSCR1 including the DNA binding domain (DNA), protein kinase C phosphorylation site (PKC), cystein-rich domain (C), nuclear localization signal (NLS), EF hands (EF), and transmembrane domain[32]. Exons are not drawn to scale. Lower panel shows human PLSCR1 products of total RNA from monocytes with exons at the right. Splice variants 1 to 3 highlighted in the right panel correspond to the 3 identified PLSCR1 variants. *Stop codon.

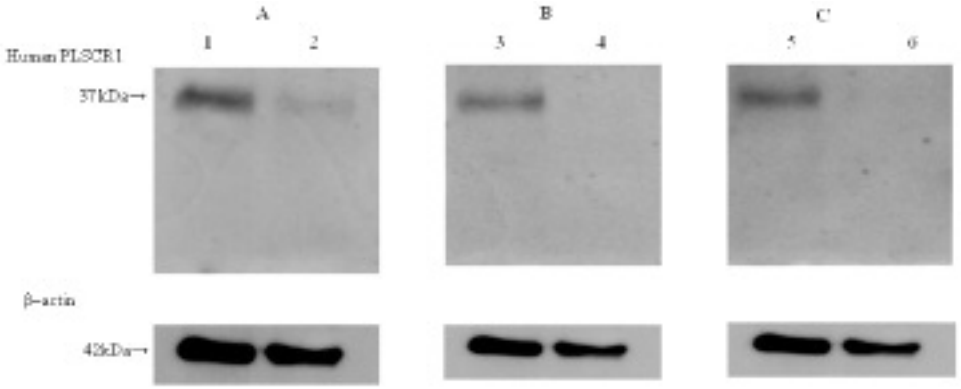

Figure 3. Western blotting shows cell lysates from peripheral blood mononuclear cells from a healthy control treated with (lanes 1, 3, and 5) or without interferon-α (IFN) (lanes 2, 4 and 6) for 24 hours. Western blotting was assayed using 3 monoclonal antibodies against human PLSCR1, (A) antibody specific for human PLSCR1 N-terminal region, (B) antibody directed against the C-terminal region of PLSCR1, and (C) antibody directed against full-length recombinant PLSCR 1 protein. A single band of 37 kDa was observed in IFN-treated cells. Lower panel shows bands corresponding to ß-actin.

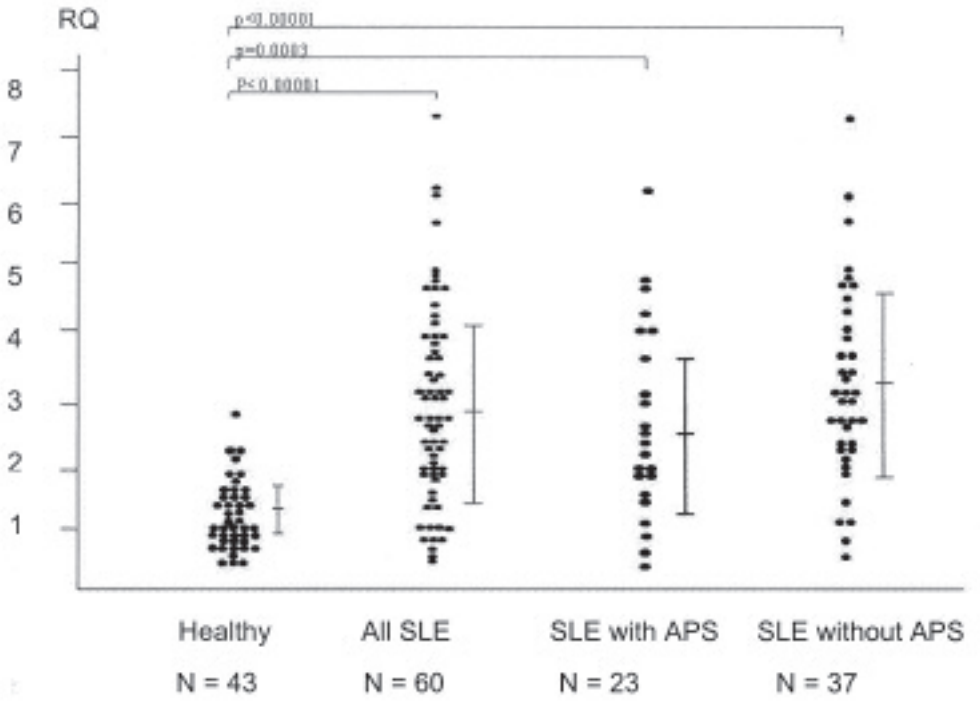

Figure 4. Quantitative real-time PCR analysis of PLSCR1. Gene expression of PLSCR1 in CD14+ cells was evaluated in healthy controls and in SLE patients with or without antiphospholipid syndrome (APS) by real-time PCR. Values were normalized to expression of the housekeeping gene GAPDH and expressed as relative quantification (RQ) in the Y-axis. Data are shown as individual results. Horizontal lines show the mean ± SD. PLSCR1 mRNA expression was significantly higher in patients with SLE.

surface was increased in CD14+ cells from SLE patients compared to controls (Table 2). No statistically significant correlations were found between the levels of PS expression and the autoantibodies investigated above or with SLEDAI/CRP levels.

In addition, no statistically significant correlation was observed between PLSCR1 mRNA levels and PS expression on CD14+ cells in patients with SLE.

DISCUSSION

In our study, we showed enhanced fibrin turnover in patients with SLE without acute thrombosis, presumably related to the prothrombotic tendency seen in such patients. A history of thrombosis was found in 23 SLE patients, which, together with increased fibrin turnover represented by elevated D-dimer plasma levels, is in concordance with the epidemiological observation of increased frequency of thrombotic events in SLE[3].

Table 2. Exposure of phosphatidylserine on the cell surface. Values are mean ± SD of percentage of annexin V high-binding cells.

Group		p
Healthy subjects, n = 24	17.8 ± 5.8	
SLE patients, n = 29	25.7 ± 11.6	1 vs 2, 0.003
2a APS, n = 7	20.6 ± 8.2	1 vs 2a, NS
2b non-APS, n = 22	27.3 ± 12.2	1 vs 2b, 0.003

SLE: systemic lupus erythematosus, APS: antiphospholipid syndrome. NS: not statistically significant.

Several studies show that SLE patients are at high risk of thromboembolic disease. The presence of aPL, other thrombotic risk factors, and predisposing conditions may contribute to the pathogenesis of the thrombophilic state in SLE, but the underlying disease mechanisms of the thrombotic tendency in SLE are not yet completely clarified[3,19,20,21,22,23].

Cell activation leads to rapid redistribution of cell membrane phospholipids and the appearance of PS on the cell surface. One of the main enzymes responsible for the externalization of PS is PLSCR1, and we speculate that SLE patients may have some abnormality in the regulation of PS expression by PLSCR1.

We investigated PLSCR1 mRNA expression in monocytes and identified 3 novel splice variants in PLSCR1, regardless of the presence of SLE. Human PLSCR1 gene is located on chromosome 3q23 and spans about 30 kb with 9 exons. Our sequencing analysis revealed 3 splicing variants generated through exon 4, exons 4/6, or exons 4/6/7 splicing out, respectively. Although these splice variants do not correspond to new isoforms of PLSCR1 protein, their identification is essential for accuracy in the quantification of PLSCR1 mRNA expression by PCR-based methods. Recently, Bernales, *et al*[24] evaluated the gene expression of PLSCR1 mRNA in PBMC from 12 SLE and 7 primary APS patients, and found higher expression of PLSCR1 mRNA in primary APS patients. We also analyzed PLSCR1 mRNA expression in monocytes from 17 primary APS patients (data not shown), and observed enhanced levels in SLE compared to those in primary APS patients. Moreover, the presence of APS in SLE patients did not affect PLSCR1 mRNA levels. The discrepancy in these data may be due to the different methodology used for PLSCR1 mRNA quantification, or to patient variables that may influence expression of PLSCR1. Bernales, *et al*[24] used a pair of primers that recognized the junction sequence between exons 1 and 2, leading to quantification of the full-length PLSCR and the 3 spliced mRNA variants. In contrast, our primer selection recognized the junction sequence between exons 3 and 4, resulting in quantification of full-length PLSCR1 mRNA expression only. Our RT-PCR results showed that the intensities of each splice variant differed among individuals, but there was no specific pattern in the band intensity in patients with SLE. Because the variants did not turn to the protein, the biological significance of splice variants was not specified. Therefore, we did not quantify the splice variants themselves.

In our study, SLE patients had elevated expression of PLSCR1 mRNA in circulating procoagulant cells, as well as high plasma levels of D-dimer in the absence of lupus activity and acute thrombosis, suggesting the prothrombotic state in SLE as a baseline. We had predicted a correlation between lupus activity and monocyte variables, but in fact SLEDAI scores did not correlate significantly with them. We consider that PLSCR1 upregulation is due to total biological alteration, which generally occurs in patients with SLE, but not to particular factors such as aPL.

PLSCR1 was reconstituted from platelets and erythrocytes[25,26], but has also been detected in a variety of cells[10]. Considering procoagulant cell activation in patients with SLE, it is likely that PLSCR1 overexpression in other procoagulant cells occurs in lupus patients.

PLSCR1 expression is induced by IFN[27], or by various growth factors[28,29,30]. Kirou, *et al*[31] showed that expression of IFN-α-inducible genes was significantly higher in PBMC from SLE than in those from controls, indicating IFN-α is the predominant stimulus for those inducible genes in SLE. Increased PLSCR1 expression may be related to IFN-α upregulation in SLE patients. Upregulation of IFN-α is considered to be linked to the inflammation and autoimmunity characteristic of SLE, and possibly being extended to thrombophilia through overexpression of PLSCR1. Our SLE patients had high fibrin turnover regardless of the presence of aPL. However, the thromboembolic complications in SLE are more common in patients with aPL, pointing to some regulator mechanisms that counterbalance the prothrombotic tendency. Antiphospholipid antibodies may affect those regulator mechanisms and represent an aggressive driver to promote thrombosis in SLE.

We demonstrated that PS exposure is relatively increased in the surface of monocytes in patients with SLE compared with healthy controls, but we failed to demonstrate the linear correlation between PLSCR1 mRNA levels and monocyte PS expression. The exposure of PS on monocytes is likely related to multiple mechanisms such as the inhibition of an ATP-dependent aminophospholipid translocase activity, an enzyme responsible for the maintenance of membrane phospholipid asymmetry in quiescent cells, and other processes required to maintain the integrity of the membrane. PLSCR1 is not the sole determinant of PS externalization and the balance between aminophospholipid translocase and PLSCR1 activities may ultimately determine the appearance of PS on the cell surface. PLSCR1 is, in any case, a strong driver of PS externalization.

We identified novel splice variants of PLSCR1. Monocytes in SLE patients had increased PLSCR1 mRNA expression, suggesting that PLSCR1 is one of the contributing factors in the prothrombotic tendency in SLE. Apart

from our findings of enhanced PLSCR1 mRNA in monocytes, multiple potential mechanisms of thrombosis, such as atherosclerosis, may affect the prothrombotic state in patients with SLE.

Further investigation into the mechanisms and biological significance of impaired PLSCR1 expression will contribute to better management of affected patients.

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