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Proteomic Analysis in Monocytes of Antiphospholipid Syndrome Patients

Deregulation of Proteins Related to the Development of Thrombosis

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Objective. **Antiphospholipid antibodies (aPL) are closely related to the development of thrombosis, but the exact mechanism(s) leading to thrombotic events remains unknown. In this study, using proteomic techniques, we evaluated changes in protein expression of monocytes from patients with antiphospholipid syndrome (APS) related to the pathophysiology of the syndrome.**

Methods. **Fifty-one APS patients were included. They were divided into 2 groups: patients with previous thrombosis, and patients with recurrent spontaneous abortion. As controls, we studied patients with thrombosis but without aPL, and age- and sex-matched healthy subjects.**

Results. **The proteins that were more significantly altered among monocytes from APS patients with thrombosis (annexin I, annexin II, protein disulfide isomerase, Nedd8, RhoA proteins, and Hsp60) were functionally related to the induction of a procoagulant state as well as to autoimmune-related responses. Proteins reported to be connected to recurrent spontaneous abortion (e.g., fibrinogen and hemoglobin) were also determined to be significantly deregulated in APS patients without thrombosis. In vitro treatment with IgG fractions purified from the plasma of APS patients with thrombosis changed the pattern of protein expression of normal monocytes in the same way that was observed in vivo for monocytes from APS patients with thrombosis.**

Conclusion. **For the first time, proteomic analysis has identified novel proteins that may be involved in the pathogenic mechanisms of APS, thus providing potential new targets for pathogenesis-based therapies for the disease.**

Antiphospholipid syndrome (APS) is an acquired autoimmune disorder of unknown pathogenesis that is defined by the association of arterial or venous thrombosis and/or pregnancy morbidity in the presence of antiphospholipid antibodies (aPL), i.e., anticardiolipin antibodies (aCL) and lupus anticoagulant (LAC) (1). Antiphospholipid antibodies are a heterogeneous family of autoantibodies whose origin and role are not fully understood. Many of these autoantibodies are directed against phospholipid-binding plasma proteins, such as β_2-glycoprotein I (β_2GPI) and prothrombin, or phospholipid–protein complexes, located on the surface of vascular endothelial cells, platelets, or monocytes (2). Several nonexclusive mechanisms could explain the involvement of aPL in the pathogenesis of thrombosis in APS, including the induction of tissue factor (TF) expression by endothelial cells and monocytes (3,4). Intracellular mechanisms underlying aPL-induced TF gene and protein expression in endothelial cells and monocytes have also been delineated at a molecular level

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(5,6). Nevertheless, despite these findings, the precise pathogenesis of thrombotic diathesis associated with aPL remains unknown.

Genetic factors related to aPL and APS have been widely investigated. Many candidate genes, including HLA class II haplotype, predispose patients to APS (7). However, genetic risk factors related to aPL development and clinical manifestations of APS in these patients remain elusive because of the heterogeneity in antigen specificity and in the pathophysiology of thrombosis. Moreover, gene expression analysis does not cover the entire spectrum of proteins produced by the organism. Due to posttranscriptional and posttranslational modifications, as well as genomic rearrangements (in the case of Ig), the number of proteins greatly exceeds the number of genes. Therefore, expression analysis does not allow the identification of changes in small subsets of Ig or T cell receptors, which may lead to the production of autoreactive T cells that contribute to the development of autoimmune diseases. Thus, the aim of our study was to investigate changes in the proteomic patterns of monocytic cells that could underlie the pathogenic mechanisms associated with thrombosis in this autoimmune disease. These studies have helped identify critical proteins that might be involved in the pathogenesis of APS.

PATIENTS AND METHODS

Patients. Fifty-one patients (42 women [none pregnant] and 9 men; mean age 43 years [range 18–74]) who fulfilled the classification criteria for APS (1) were included in the study after ethics committee approval was obtained. All patients provided written informed consent. They were divided into 2 groups: group 1 consisted of 32 patients with previous thrombotic events (arterial in 20 patients [62.5%] and venous in 12 patients [37.5%]), and group 2 consisted of 19 patients with recurrent spontaneous abortion. We excluded all APS patients with evidence of an underlying systemic rheumatic disease or antibodies against double-stranded DNA or extractable nuclear antigen. Patients were studied for at least 9 months after their latest thrombotic event or spontaneous abortion. As controls, we studied 20 patients with verified thrombosis (8) but without aPL (group 3; venous in 10 patients [50%] and arterial in 12 patients [58%]), and 15 healthy subjects (group 4). None of the healthy controls had a history of autoimmune disease, bleeding disorders, thrombosis, or miscarriage. None of the patients had protein C, protein S, or antithrombin deficiency, factor V Leiden, or prothrombin G20210A.

Patients were tested for the presence of aCL and LAC (9,10). The results for aCL were expressed in IgG and IgM phospholipid units (GPL and MPL units, respectively), and were reported as positive if they were present in medium or high titers (>40 GPL units) (1). IgG and IgM aCL were positive in 33 and 28 APS patients, respectively, and LAC was positive in 36 patients.

All APS patients with thrombosis were being treated with an oral anticoagulant (dicumarol). APS patients without thrombosis were taking a low dosage of aspirin (125 mg/day) or received no treatment. Finally, some patients with thrombosis but without APS were taking oral anticoagulants and some received no treatment. No patients were being treated with immunomodulatory agents, since they had no other underlying systemic autoimmune disease.

Monocyte isolation. Peripheral venous blood samples were collected in sterile precooled tubes containing 0.129*M* sodium citrate (1/9 [volume/volume]; Becton Dickinson, Meylan, France) and centrifuged immediately at 500*g* for 10 minutes at 4°C to remove platelets. Monocytes were isolated by depletion of nonmonocytes, using a commercial kit (Miltenyi Biotech, Bergisch Gladbach, Germany), which enabled us to obtain monocytes without activation (6). Briefly, peripheral blood mononuclear cells (PBMCs) were obtained by density gradient centrifugation over Ficoll-Paque. PBMCs were depleted from T cells, natural killer cells, B cells, dendritic cells, and basophils by indirect magnetic labeling with a cocktail of hapten-conjugated CD3, CD7, CD19, CD45RA, CD56, and anti-IgE antibodies and microbeads coupled to an antihapten monoclonal antibody. Magnetically labeled cells were removed by retention on a column in a magnetic field. The purity of isolated monocytes was evaluated by staining cell aliquots with a fluorochrome-conjugated antibody against monocytes (fluorescein isothiocyanate [FITC]–conjugated anti-CD14), and analyzed by flow cytometry. Using this method, a mean ± SEM 92.7 ± 3% viable monocytic cells were obtained.

Flow cytometric analysis of TF activity. Flow cytometric analysis was performed using a FACScan (BD Biosciences, San Jose, CA) (9) and specific monoclonal antibodies to human TF (clone TF9-6B4, FITC conjugated; American Diagnostica, Greenwich, CT) or to human CD14 (phycoerythrin-conjugated; Caltag, South San Francisco, CA). TF activity on intact and lysed monocytic cells was determined using a continuous chromogenic assay (9,11).

IgG purification and in vitro exposure of normal monocytes to aCL. IgG from the pooled sera of 7 patients with APS and thrombosis, and from the pooled sera of 10 healthy controls was purified by protein G–Sepharose affinity chromatography (MabTrap kit; Amersham Biosciences, Uppsala, Sweden). Endotoxin in IgG fractions was measured by *Limulus* amebocyte lysate assay (Amebo-lysate; ICN Biomedicals, Costa Mesa, CA). Anti-β_2GPI activity of purified IgG was confirmed by enzyme-linked immunosorbent assay and reported semiquantitatively in standard IgG anti-β_2GPI units. IgG and aCL were determined as described above, and titers were reported in GPL units.

For in vitro studies, normal monocytes purified from healthy controls were cultured with serum-free RPMI 1640 containing 2 m*M* L-glutamine, 100 units/ml penicillin, 100 mg/ml streptomycin, and 250 pg/ml Fungizone (BioWhittaker/MA Bioproducts, Walkersville, MD) at 37°C in a humidified, 5% CO_2 atmosphere. Monocytes (1.5×10^6/ml) were incubated for 6 hours at 37°C with purified APS patient IgG (100 μg/ml) or normal human serum IgG (obtained from healthy controls).

Protein extraction. Purified monocytes were washed twice with chilled phosphate buffered saline. Then, cell pellets were resuspended with lysis buffer (200 μl per 10^7 cells) containing 7.0*M* urea, 2*M* thiourea, 4% CHAPS, 2% carrier ampholytes, and 1% dithiothreitol (DTT) (12). Extracted proteins were separated by centrifugation at 12,000*g* for 15 minutes, and the supernatant was used for 2-dimensional (2-D) electrophoresis. Protein levels were determined using a protein assay (Bio-Rad, Hercules, CA). Samples were stored at −80°C until used.

Two-dimensional gel electrophoresis. Immobilized pH gradient strips (11 cm, range 3–10 pH; Bio-Rad) were passively rehydrated with 350 μg of protein lysate in 150 μl of rehydration buffer (7*M* urea, 2*M* thiourea, 4% CHAPS, 20 m*M* DTT, 0.5% Triton X-100, 0.5% Pharmalyte 3–10, and 0.001% bromphenol blue) for 12 hours. Isoelectric focusing (IEF) was performed at 20°C, using a Protean IEF system (Bio-Rad). Thereafter, the strips were soaked for 10 minutes in equilibration solution (50 m*M* Tris HCl, pH 8.8, 6*M* urea, 30% glycerol, 2% sodium dodecyl sulfate [SDS], and 0.001% bromphenol blue) that contained 20 mg/ml DTT and then for an additional 10 minutes in equilibration solution that contained 25 mg/ml iodoacetamide (13). The second-dimension procedure was performed in 12.5% polyacrylamide gel at 35 mA/gel (Mini-Protean 3 cell; Bio-Rad).

Detection of protein spots and data analysis. Gels were silver stained using the Plus One Protein Silver Staining kit (Amersham Biosciences). Images were obtained using a GS-800 calibrated densitometer (Bio-Rad) and analyzed with PDQuest 7.1.0 2-D analysis software (Bio-Rad). Gels were divided into the 4 groups of study, and protein spots were matched between gels (performed at least in duplicate for each patient sample) and between groups. To accurately compare spot quantities between gels, image spot quantities were normalized by dividing the raw quantity of each spot in a gel by the total quantity of all valid spots in that gel. Spots present in <50% of the control gels and in <50% of the patient gels were filtered out of the analyses. In each of the 3 patient groups, the mean value of the quantity of each remaining spot in the gel was expressed as a ratio to the mean value in control gels. Mean values of each spot on the 2 duplicate gels per individual were calculated and then compared using the Mann-Whitney 2-tailed rank sum test. Samples from healthy controls were used to generate control maps. Fluctuations in the protein expression levels among control maps were monitored using densitometry analysis, and a coefficient of variation (CV) was generated for the mean value of each spot.

Protein spots of interest were manually excised from preparative gels and subjected to mass spectrometry (MS) analysis. Peptides of each sample were analyzed in a 4700 Proteomics Station (Applied Biosystems, Foster City, CA) in automatic mode at the Proteomics Service of the University of Córdoba. After drying, samples were analyzed in the 800–4,000 mass/charge range, with an accelerating voltage of 20 kV. Spectra were internally calibrated with peptides from trypsin autolysis. Proteins were identified by peptide mass fingerprinting, and confirmed by matrix-assisted laser desorption ionization (MALDI)–time-of-flight MS. The Mascot search engine (Matrix Science, Boston, MA) was used for protein identification over the Mass Spectrometry protein sequence DataBase.

Validation of proteomic data by quantitative real-time reverse transcriptase–polymerase chain reaction (RT-PCR). Total cellular RNA from monocytic cells was extracted using TRI Reagent (Sigma, St. Louis, MO). All PCRs were performed using the LightCycler (Roche Diagnostics, Indianapolis, IN). The primer sequences used in this study and the theoretical size of the PCR products are as follows: TF forward CTACTGTTTCAGTGTTCAAGCAGTGA, reverse CAGTGCAATATAGCATTTGCAGTAGC (283 bp); Hsp60 forward 5′-ATTCCAGCAATGACCATTGC-3′, reverse 5′-GAGTTAGAACATGCCACCTC-3′ (306 bp); annexin II forward 5′-ATGTCTACTGTTCACGAAATC-3′, reverse 5′-AATGAGAGAGTCCTCGTCGG-3′ (387 bp); annexin I forward 5′-TTGAGGAGGTTGTTTTAGCTCTG-3′, reverse 5′-AGTTCTTGATGCCAAAATCTCAA-3′ (125 bp); RhoA forward 5′-CGCTTTTGGGTACATGGAGT-3′, reverse 5′-GGAGGGCTGTTAGAGCAGTG-3′ (247 bp); Nedd8 forward 5′-AGAGCGTGACCGGAAAGGA-3′, reverse 5′-TCATCATTCATCTGCTTGCCAC-3′ (142 bp); protein disulfide isomerase (PDI) forward 5′-GAATCTTTCTGAAGCCACAC-3′, reverse 5′-CATACGACCCAGAACCATC-3′ (235 bp); GAPDH forward 5′-TGATGACATCAAGAAGGTGGTGAAG-3′, reverse 5′-TCCTTGGAGGCCATGTAGGCCAT-3′ (239 bp).

One-step RT-PCR was performed using the QuantiTect SYBR Green RT-PCR kit (Qiagen, Hilden, Germany) (6). Samples of messenger RNA (mRNA) were analyzed in at least 3 similar RT-PCR procedures. Negative controls containing water instead of RNA were run to confirm that samples were not cross-contaminated. Relative expression was quantified by the standard curve method, as recommended by the manufacturer (Qiagen), and the target amount was normalized to the GAPDH gene. Expression of mRNA was considered positive in patient samples when the value was >1.7-fold higher than in controls.

Validation of proteomic data by Western blotting. Total cell lysate fractions (50 μg), prepared by standard protocols (14), were resolved by SDS–10% polyacrylamide gel electrophoresis and then transferred to nitrocellulose membranes. Annexin I, annexin II, PDI, Nedd8, RhoA, and Hsp60 protein levels were determined by Western blotting using their respective monoclonal or polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). Protein levels were quantified using the image analysis software Intelligent Quantifier, version 2.1.1 (Bio Image Systems, Jackson, MI). Results were calculated as integrated optical density and expressed in arbitrary units.

Statistical analysis. All data are expressed as the mean ± SEM, except those in Table 2, which are the mean ± SD. Statistical analyses were performed using Sigmastat software (Jandel Scientific, Erkrat, Germany). Before comparing 2 data groups, normality and equal variance tests were performed. If both tests were passed, comparison was made using a parametric test (Student's paired *t*-test). If the normality and/or equal variance test was violated, a nonparametric test (Mann-Whitney rank sum test) was used instead. Groups were compared by analysis of variance. Correlations were assessed by Pearson's product-moment correlation coefficient. *P* values less than 0.05 were considered significant.

RESULTS

Titers of aCL antibodies, and TF expression and activity in monocytes from APS patients. IgG aCL antibody isotypes were positive in 25 of the 32 patients with primary APS with thrombosis (mean ± SEM 83.3 ± 10.1 GPL units) and in 8 of the 19 patients with primary APS without thrombosis (57.2 ± 7.9 GPL units). IgM aCL antibody isotypes were positive in 17 of the 32 patients with primary APS with thrombosis (48.9 ± 12.3 GPL) and in 11 of the 19 patients with primary APS without thrombosis (75.3 ± 11.6 GPL).

Mean TF mRNA levels were significantly higher in monocytes from patients with a history of thrombosis (relative expression level 3.57 ± 0.6) than in those from patients without thrombosis (1.57 ± 0.3; $P < 0.025$), from patients with thrombosis but without APS (1.68 ± 0.4; $P < 0.035$), and from healthy controls (0.59 ± 0.1; $P < 0.001$). Accordingly, cell surface–associated TF was significantly increased in patients with thrombosis (49.7 ± 3.8% positive cells) compared with patients without thrombosis (22.4 ± 1.9%; $P < 0.001$), patients with thrombosis but without APS (19.9 ± 2.8%; $P < 0.001$), and with healthy controls (4.8 ± 0.8%; $P < 0.001$).

There was also a direct correlation between the degree of TF antigen expression and procoagulant activity (PCA) of TF. PCA of TF on intact cells and cell lysates was significantly higher in group 1 (157.7 ± 12.6 units/10^5 lysate cells) than in the other 3 groups (64.5 ± 12.3, 39.7 ± 7.8, and 27.4 ± 2.1 units/10^5 lysate cells in groups 2, 3, and 4, respectively; $P < 0.001$). Patients with primary APS with thrombosis showed a significant increase in TF expression among those who were positive for IgG aCL antibody isotypes ($P < 0.05$), but not among those positive for IgM aCL antibody isotypes or LAC, thus confirming the results of previous studies (9).

Proteomic findings. At least 2 separate experiments were performed on all samples, and similar protein spot patterns were obtained. Approximately 500 protein spots were detected on silver-stained gels. Nearly 85% of all spots were matched on duplicate gels, and the intensity of the same spot on different gels showed no significant changes. Representative gels of the highly expressed proteins in control and APS monocytes are shown in Figure 1. We identified 29 spots in APS monocytic cells, of which 22 corresponded to known proteins, whose expression was altered significantly between monocytes from APS patients with thrombosis and the remaining groups ($P < 0.05$). These identified proteins are listed in Table 1.

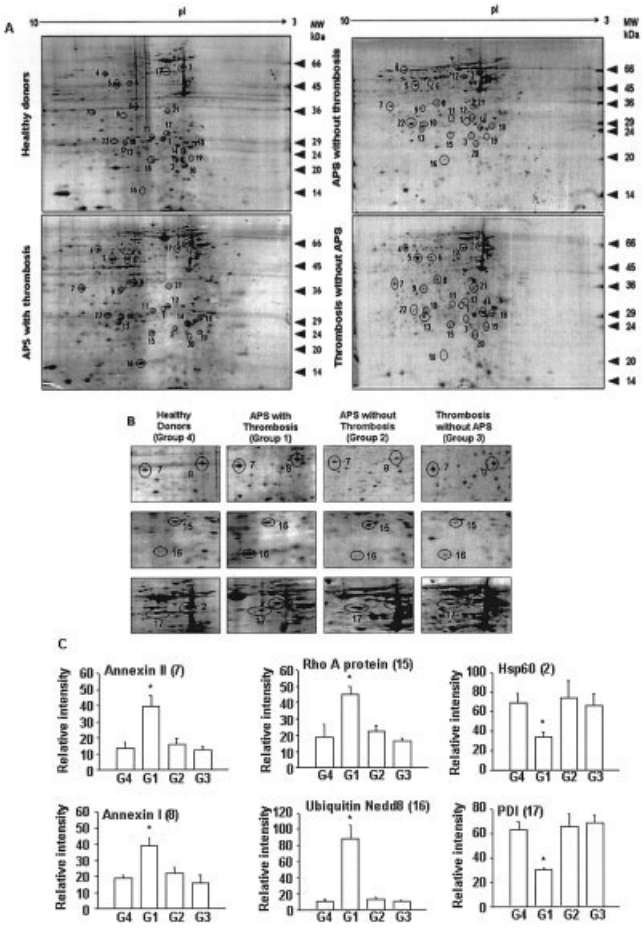

Figure 1. Representative gels of the highly expressed proteins in monocytes from the 4 groups (G) analyzed. **A,** Scanned images of the silver-stained gels used to detect and compare spots. The positions of proteins that were differentially expressed between groups are circled and indexed as numbered. Molecular mass standards are shown on the right. **B,** Representative close-up views of the differentially expressed protein spots in the 4 groups analyzed, as indicated in 2-dimensional echocardiogram maps. **C,** Relative normalized amounts of identified proteins. Values are the mean and SEM. $* = P < 0.05$ versus healthy donors. The complete list of proteins is shown in Table 1. APS = antiphospholipid syndrome; PDI = protein disulfide isomerase.

The proteins identified as being more significantly altered between monocytes from APS patients with thrombosis (group 1) and the remaining groups were annexin I, annexin II, PDI, Nedd8, RhoA, and Hsp60. These proteins were functionally related to processes associated with the induction of a procoagulant state, as well as autoimmune-related responses. Furthermore, we found a significant correlation between the titers of IgG aCL and the expression levels of the above-mentioned proteins (all $P < 0.05$). Variability of

Table 1. Proteins differentially expressed in monocytes from APS patients with thrombosis and from healthy subjects*

Spot no.	Accession no.†	Protein name	Functional classification	Peptide coverage, %	Molecular mass, kd‡	pI‡	Ratio§
1	P30040	ERp28	Metabolism, response to stress	25	29.0	6.8	+1.8
2	P10809	Hsp60 I	Autoimmune responses, signal transduction	45	59.9	5.5	−2.5
3	P08263	Glutathione transferase chain A	Cell defense	18	21.8	5.7	−1.7
4	P02675	Fibrinogen precursor	Blood clot formation	29	56.6	8.5	+0.6
5	P06733	α-enolase	Energy metabolism	73	47.3	6.9	+1.9
6	P04040	Catalase	Cell defense	68	59.9	6.7	+2.2
7	P07355	Annexin II	Receptor for aPL induction of cell activation	59	38.5	7.6	+2.4
8	P04083	Annexin I	Macrophage activity, immune responses, ERK activation	48	38.9	6.6	+2.6
9	P40925	Malate dehydrogenase	Metabolism	21	36.5	6.9	+1.4
10	Q8NOY7	Phosphoglycerate mutase 4	Energy metabolism	19	28.9	6.2	+1.7
11	P74817	Glutathione transferase O1-1	Energy metabolism	27	27.8	6.2	+1.5
12	Q06323	PA28, α chain	Signal transduction	35	28.9	5.8	+1.4
13	P60174	Triosephosphate isomerase	Metabolism	58	26.8	6.5	+1.9
14	P02647	Apolipoprotein A-I	Metabolism	27	30.7	5.6	+2.3
15	P61586	RhoA	Modulation of macrophage gene/protein expression, signal transduction	65	21.8	5.8	+3.8
16	Q15843	Nedd8	NF-κB activation	53	8.4	6.9	+8.9
17	P07237	PDI	Immune and inflammatory responses	48	56.7	6.0	−3.7
18	P52566	Rho GDIβ	Signal transduction	52	22.8	5.1	+1.5
19	P06727	Apolipoprotein A-IV precursor	Metabolism	18	34.4	5.7	+1.4
20	P51812	RS6K 3 (fragment)	Signal transduction	23	29.0	6.0	−1.9
21	Q86X76	NIT 1 protein	Metabolism	16	26.9	5.8	+0.7
22	P0242	Hemoglobin, chain D	Metabolism	23	15.9	7.9	+1.4

* ERp28 = endoplasmic reticulum protein 28; aPL = antiphospholipid antibody; PA28 = proteasome activator 28; PDI = protein disulfide isomerase; RS6K = ribosomal S6 kinase.
† For the identification of the proteins, the repositories used were the Swiss-Prot and TrEMBL databases.
‡ Molecular mass and pI denote experimental values.
§ Expression in antiphospholipid syndrome (APS) patients with thrombosis in relation to the expression in healthy donors. Positive ratios denote up-regulated proteins; negative ratios denote down-regulated proteins.

the differentially expressed protein spots among the 10 controls used was very low, as demonstrated by CV analysis. The proteins that were more significantly altered in monocytes from patients with primary APS with thrombosis compared with the other groups are shown in Figures 1A and B and are listed in Table 2.

In addition, proteins associated with metabolism, protein folding/modification, immune response, and transcriptional factors were readily identified as being distinctively expressed in monocytes from APS patients with thrombosis compared with the remaining groups analyzed. No significant differences were seen in the expression levels of those proteins between monocytes from healthy subjects and APS patients without thrombosis, between healthy subjects and patients with thrombosis but without APS, or between APS patients without thrombosis and patients with thrombosis but without APS, with the exception of 2 relevant proteins previously reported to be connected to recurrent spontaneous abortion, fibrinogen (spot 4), significantly reduced versus the remaining groups ($P < 0.05$), and hemoglobin (spot 22), significantly increased versus the remaining groups ($P < 0.05$). Both of these proteins were found to be significantly deregulated in APS patients without

Table 2. Expression levels (versus those in healthy donors) of the 6 proteins found to be highly differentially expressed in monocytes from patients with primary APS with thrombosis*

Protein	Mean ± SD % change in protein expression vs. healthy donors: Primary APS with thrombosis	Primary APS without thrombosis	Thrombosis without APS	CV in monocytes, %
Annexin I	180.6 ± 37.5	97.4 ± 20.3	112.7 ± 5.4	22.3
Annexin II	220.6 ± 51.5	105.3 ± 30.1	97.4 ± 11.2	18.5
PDI	22.7 ± 5.91	88.7 ± 12.8	93.2 ± 10.9	12.1
Nedd8	887.1 ± 65.1	101.2 ± 18.8	98.5 ± 19.1	15.2
RhoA	378.2 ± 47.6	127.4 ± 15.0	123.1 ± 29.2	20
Hsp60 1	52.2 ± 8.4	88.7 ± 10.2	92.4 ± 16.2	14.2

* CV = coefficient of variation (see Table 1 for other definitions).

thrombosis compared with the other groups of patients studied ($P < 0.05$).

Western blot and RT-PCR analyses of differentially expressed proteins in APS patients with thrombosis. To validate 2-D data, annexin I, annexin II, PDI, Nedd8, RhoA, and Hsp60 levels were analyzed by Western blotting (Figures 2A and B). These results showed that all 6 proteins were correctly identified by MALDI tandem MS.

RT-PCR analysis was performed on those 6 genes identified by proteomic analysis (Figure 2C). RT-PCR analysis confirmed the proteomic data for 4 of these (RhoA, annexin II, PDI, and Hsp60), but mRNA levels were not consistent with the data obtained for the remaining 2 (annexin I and Nedd8) by differential proteomic analysis and Western blotting. These data strongly suggest that, for a number of the identified proteins, differences that exist between the 2 cell systems may not be due to altered gene expression, but rather to alterations of other control mechanisms, such as mRNA stability or lifespan, translation efficiency, posttranslational processing, control of protein turnover, or a combination of these (15,16).

Changes induced by aPL in the proteomic profile of normal monocytes. All IgG aPL samples from the 7 patients were positive for aCL (>100 GPL units), and all IgG samples from the healthy controls were negative for aCL (<10 GPL units). The mean ± SD anti-β_2GPI antibody activities of the IgG aPL and control IgG were 78.4 ± 10.7 GPL units and 4.7 ± 0.8 GPL units, respectively. All patient IgG selected for this study had moderate to high anti-β_2GPI activity, which correlated with the level of IgG ($P = 0.0375$, r = 0.917). All IgG preparations tested negative for lipopolysaccharide in the *Limulus* amebocyte lysate assay. To ascertain that changes observed in proteins related to thrombotic events in APS patients were directly dependent on aPL-induced activation, we compared the effects of patient IgG and control IgG on the expression of annexin I, annexin II, PDI, Nedd8, RhoA, and Hsp60. Our results showed that IgG fractions purified from the plasma of APS patients with thrombosis changed the protein expression pattern of normal monocytes in the same way that was observed in vivo for monocytes from APS patients with thrombosis (Figures 3 and 4). These

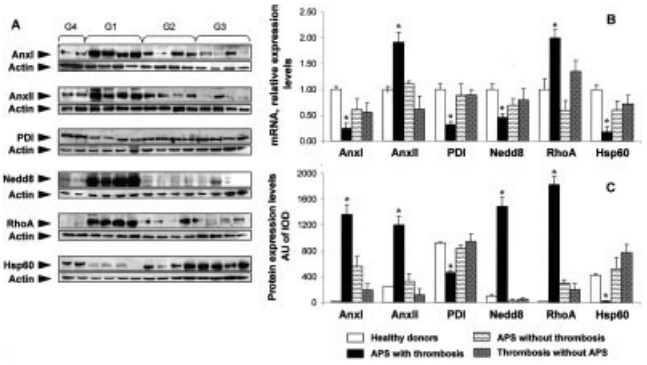

Figure 2. Validation of proteomic data by Western blotting and real-time reverse transcriptase–polymerase chain reaction. **A,** Representative Western blots of 4 samples from each patient group (G) and 2 control samples, performed in triplicate, as described in Patients and Methods. **B** and **C,** Mean and SEM mRNA and protein expression levels of the 6 proteins identified by proteomic analysis in the 4 groups of patients and controls. ∗ = $P < 0.05$ versus healthy donors. AnxI = annexin I; AU = arbitrary units; IOD = integrated optical density (see Figure 1 for other definitions).

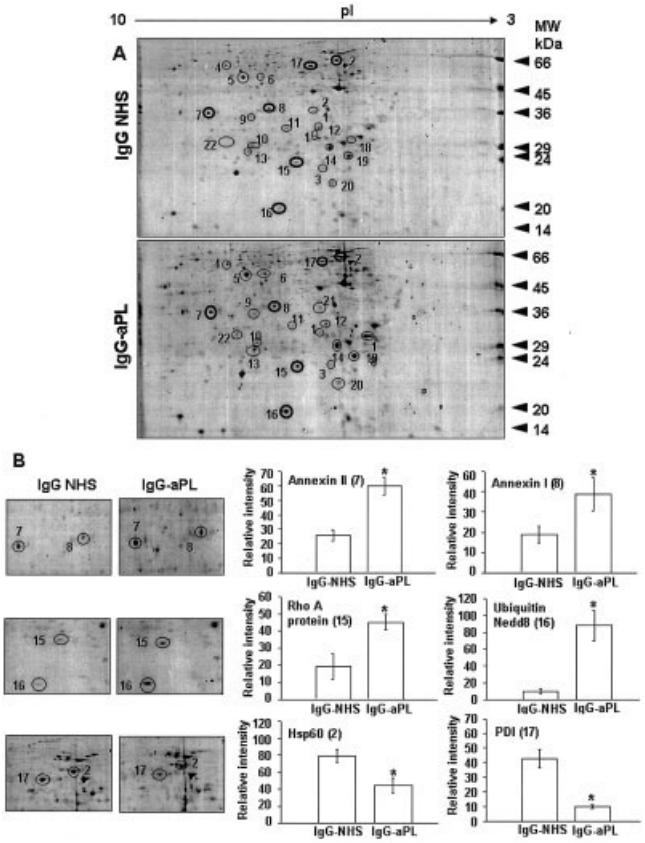

Figure 3. Antiphospholipid antibody (aPL)–induced changes in the proteomic profile of normal monocytes. **A,** Representative gels of differentially expressed proteins in monocytes treated with normal human serum (NHS) IgG or antiphospholipid syndrome patient IgG. **B,** Representative close-up views of the differentially expressed protein spots, and relative normalized amounts of these proteins. Values are the mean ± SEM. ∗ = $P < 0.05$ versus controls. AnxI = annexin I; PDI = protein disulfide isomerase.

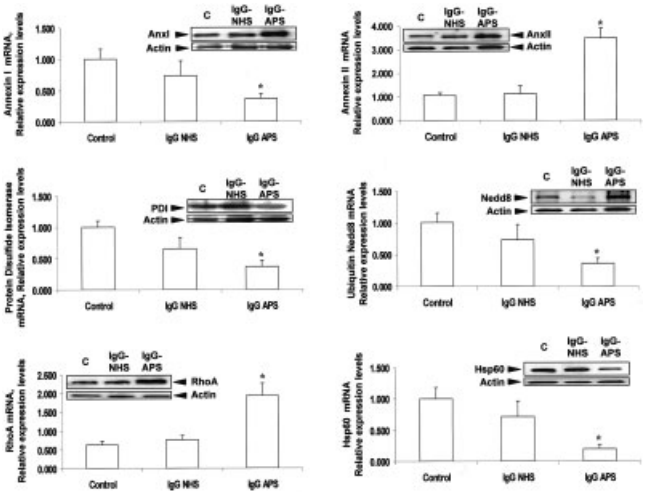

Figure 4. Validation of proteomic data from in vitro studies by Western blotting and real-time reverse transcriptase–polymerase chain reaction. Values are the mean and SEM mRNA expression levels of the 6 proteins differentially expressed after in vitro treatment with normal human serum (NHS) IgG or antiphospholipid syndrome (APS) patient IgG, as identified by proteomic analysis. A representative Western blot of each protein, analyzed in 3 independent experiments with similar results, is also shown. $* = P < 0.05$ versus controls. AnxI = annexin I; PDI = protein disulfide isomerase; C = control.

IgG fractions from APS were previously shown to activate human monocytes (4,6,17).

DISCUSSION

Several mechanisms may contribute to thrombotic manifestations of APS. Among these, activation of monocytic cells, with the attendant loss of anticoagulant and the gain of procoagulant functions, is likely to be important. We and others have previously demonstrated that monocytes are involved in the thrombotic state characteristic of most APS patients (4,6,9). However, no study has evaluated the changes in the proteomic patterns that could underlie the pathogenic mechanisms associated with thrombosis in this disease. Here, we have identified a pattern of 22 differentially expressed proteins that is characteristic for monocytes of APS patients when compared with healthy individuals. Moreover, some of these proteins play a potential role in thrombosis development in APS (18).

Two annexins were up-regulated in monocytic APS samples: annexin I and annexin II. Annexins are a family of phospholipids and calcium-binding proteins that modulate inflammation, immune response, and blood coagulation. Annexin I may play a role in the regulation of macrophage activity, and its levels are raised in some autoimmune diseases (19,20), thus supporting the notion of its involvement in the regulation of the immune system. Also, annexin I is a substrate of protein kinase C and protein–tyrosine kinases. Increased annexin I expression leads to constitutive activation of ERK1/2 in RAW 264.7 macrophage cell lines (21). Accordingly, up-regulation of annexin I in APS monocytic cells was accompanied by constitutive activation of the MEK/ERK pathway (López-Pedrera C: unpublished observations). Thus, the multifactorial downstream effects of the action of annexin I, including antiproliferative and antiinflammatory effects, might be translocated by the ERK signaling pathway.

Annexin II is a receptor for fibrinolytic activation localized on the cell surface of endothelial cells, monocytes, and syncytiotrophoblasts (22). Annexin II is directly involved in the pathogenesis of APS. Binding of β_2GPI to human umbilical vein endothelial cells is mediated by annexin II (23). By functioning as a receptor for β_2GPI, annexin II is a target not only for anti–annexin II antibodies but also for anti-β_2GPI antibodies, which are direct inducers of TF overexpression and thus are significantly associated with thrombosis in the setting of APS (18). Recently, it has also been demonstrated that annexin II plays an important role in human monocyte/macrophage-directed migration and recruitment and that it is activated on progression from monocytes to macrophages (24). Thus, annexin II might constitute a common receptor for aPL induction of monocyte activation.

TF-induced expression by aPL in monocytes might be responsible for the prothrombotic state of APS patients (6). A recent study has shown that the surface-accessible, extracellular Cys186–Cys209 disulfide bond of TF is critical for coagulation, and that PDI (a multifunctional protein catalyzing the oxidation, reduction, and isomerization of disulfide bridges) disables coagulation by targeting this disulfide. PDI is associated with TF on the cell surface when coagulant activity is low and TF-VIIa signaling is enabled. Moreover, decreased PDI expression was associated with a 2-fold increase in TF procoagulant activity (25). Thus, reduced expression of PDI in monocytes from APS patients with thrombosis might contribute to their prothrombotic state. Additionally, our in vitro results further suggest that its down-modulation is promoted by aPL, thus connecting this protein to the mechanisms of thrombosis associated with the syndrome. Moreover, overexpression of PDI suppresses NF-κB–dependent transcriptional activity (26). Because aberrant activation of the NF-κB pathway likely contributes to the development and progression of APS,

it could be speculated that the reduced monocyte PDI expression might be related to the constitutive activation of NF-κB in APS.

Nedd8 was significantly increased in monocytic APS cells. This protein is involved in the proteolytic destruction of IκB (27). A constitutive NF-κB binding activity has been demonstrated in monocytes from APS patients in vivo, which was related to aPL-induced TF expression (6,28), as previously demonstrated in aPL-induced NF-κB activation of endothelial cells (5,29,30). Thus, increased expression of Nedd8 might account for constitutive NF-κB–binding activity in APS monocytes.

RhoA was also significantly increased in the APS with thrombosis group. RhoA proteins are modulators of gene expression, adhesion, and migration of activated macrophages, which also play critical roles in inflammatory signal pathways, such as those required for activation of NF-κB (31). In addition, inhibition of Rho/Rho kinase proteins down-regulates the synthesis of TF by cultured human monocytes, and statins (known immunoregulatory and antithrombotic compounds that are now being experimentally tested in APS patients) suppress the synthesis of TF mediated by inhibition of Rho activity (32). Agonists reported to activate Rho proteins in vascular cells include thrombin, endothelin 1, and angiotensin. Our study further indicates that aPL also contribute to their increased expression, thus suggesting that RhoA proteins may be directly involved in monocyte APS function.

Hsp60 was down-regulated in monocytes from APS patients. Hsp60 is a target of autoantibodies and autoimmune T cells in healthy individuals, as well as in those with autoimmune diseases (33). Our results are consistent with the significant reduction in Hsp60 levels in PBMCs from rheumatoid arthritis patients (34). Actually, Hsp60 contributes to the suppression of arthritis by stimulating regulatory suppressive T cells (35). On the other hand, although Hsp60 expression increases as a consequence of an inflammatory response, monocyte activation and thrombosis in APS have been proven to not be related to an acute inflammatory response (9). That might explain why in our study Hsp60 was not overexpressed, and was even reduced, both in monocytes from APS patients with thrombosis and in aPL-treated monocytic cells. Nevertheless, the underlying mechanisms explaining this reduced expression remain to be tested.

We should also highlight the identification of 2 proteins (fibrinogen and hemoglobin) that might be related to the pathogenesis of recurrent spontaneous abortion in APS. We found that the expression levels of fibrinogen in patients with APS without thrombosis were lower than in the other study groups. Recent studies have suggested that the absence or a significant decrease in maternal fibrinogen is sufficient to cause rupture of vasculature, affecting embryonic trophoblast infiltration, and leading to hemorrhagic miscarriage (36). Thus, deficiencies of fibrinogen during gestation may lead to abnormal fetal growth or abortion.

Similarly, a recent study has shown increased gene expression of hemoglobin in patients with recurrent abortion (37). Our data at the protein level further support this increase. Therefore, these proteins might be helpful in understanding the molecular mechanisms involved in recurrent abortion in APS. Recent investigations have shown that in many women with APS who had miscarriages, thrombosis was not evident in the placentae. Moreover, a very recent study (38) has shown that inflammation, specifically activation of complement with generation of the anaphylatoxin C5a, is an essential trigger of fetal injury, and that TF expression on neutrophils, but not macrophages, is essential to the pathogenesis of aPL-induced fetal loss, and reveals a functional linkage between C5a, neutrophil activation, and fetal injury. Collectively, these data might explain why our proteomic studies of monocytes from APS patients without thrombosis did not reflect many significant changes in protein expression, particularly in those proteins related to procoagulant events.

Gene expression patterns of PBMCs from APS patients have recently been analyzed, and candidate clusters of genes that clearly exhibited reliable discriminatory patterns between the APS and non-APS patient populations with thrombosis were identified (39). Notably, both apoH (β_2GPI) and MEKK1 genes, previously described in the molecular pathogenesis of APS, were included in the list of genes found to differ between APS and non-APS patients with thrombosis. Our proteomic studies further complement the genetic ones, and thus both proteomic and transcriptomic approaches may provide distinct but complementary views in profiling gene expression.

In summary, this study has identified altered expression of proteins that might be directly related to thrombotic events in APS. The development and use of such proteomic biomarkers for diagnosis, assessing prognosis, and guiding therapy might revolutionize the care of patients with APS.

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AUTHOR CONTRIBUTIONS

Dr. López-Pedrera had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. López-Pedrera, Cuadrado, Velasco, Khamashta.

Acquisition of data. Hernández, Buendía, Aguirre, Barbarroja, Torres, Khamashta.

Analysis and interpretation of data. López-Pedrera, Cuadrado, Villalba, Khamashta.

Manuscript preparation. López-Pedrera, Cuadrado, Villalba, Khamashta.

Statistical analysis. Aguirre, Barbarroja, Velasco.

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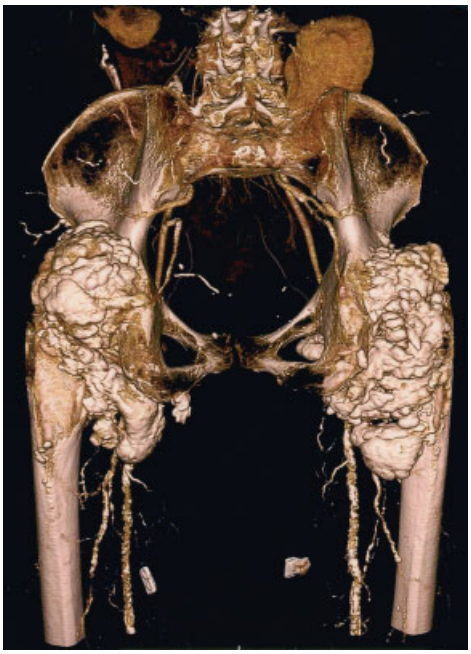
Clinical Image: Stalagmite hips in a patient with systemic sclerosis



The patient, a 61-year-old woman with a 25-year history of systemic sclerosis, presented to our clinic with chronic bilateral hip pain and severe restriction of movement. Three-dimensional computed tomographic angiography, performed to assess joint replacement as a treatment approach, provided a vivid illustration of ectopic calcifications that had developed in the course of the disease. The posteroanterior view of the pelvis presented here reveals entrapment of both hip joints within massive calcific tissue. Calcifications developed in the periarticular space and within adjacent muscles (quadriceps, gluteal muscles, obturators). Bilateral loss of cartilage space and erosion of the external side of the acetabulum were also documented, especially on the right side. Ileac arteries and deep and superficial femoral arteries were unaffected, although smaller branches appeared to be trapped within these enormous calcific masses. The morphology of ectopic calcifications in this patient resembled the formation of stalagmites.

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