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Differential Expression of Protease-Activated Receptors in Monocytes From Patients With Primary Antiphospholipid Syndrome

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Objective. **To investigate the expression of protease-activated receptors (PARs), their potential regulation by anticardiolipin antibodies (aCL), and their association with the expression of other molecules relevant to thrombosis in monocytes obtained from 62 patients with primary antiphospholipid syndrome (APS).**

Methods. **Monocytes were isolated from peripheral blood mononuclear cells by magnetic depletion of nonmonocytes. Expression of tissue factor (TF) and PARs 1–4 genes was measured by quantitative real-time reverse transcription–polymerase chain reaction. Cell surface TF and PARs 1–4 expression was analyzed by flow cytometry. For in vitro studies, purified normal monocytes were incubated with purified APS patient IgG, normal human serum IgG, or lipopolysaccharide, in the presence or absence of specific monoclonal antibodies anti–PAR-1 (ATAP2) or anti–PAR-2 (SAM11) to test the effect of blocking the active site of PAR-1 or PAR-2.**

Results. **Analysis of both mRNA and protein for the 4 PARs revealed significantly increased expression of PAR-2 as compared with the control groups. PAR-1 was significantly overexpressed in APS patients with thrombosis and in the control patients with thrombosis but without APS. PAR-3 expression was not significantly altered. PAR-4 expression was absent in all groups analyzed. In addition, we demonstrated a correlation between the levels of PAR-2 and the titers of IgG aCL, as well as parallel behavior of TF and PAR-2 expression. In vitro, IgG from APS patients significantly increased monocyte expression of PAR-1 and PAR-2. Inhibition studies suggested that there was direct cross-talk between TF and PAR-2, such that inhibition of PAR-2 prevented the aCL-induced expression of TF.**

Conclusion. **These results provide the first demonstration of increased expression of PARs in monocytes from patients with APS. Thus, PAR antagonists might have therapeutic potential as antithrombotic agents in APS.**

The antiphospholipid syndrome (APS) is an acquired autoimmune disorder of unknown cause. The syndrome is defined by the association of arterial and/or venous thrombosis and/or pregnancy morbidity in the presence of antiphospholipid antibodies (aPL): anticardiolipin antibodies (aCL), lupus anticoagulant (LAC), and anti–β_2-glycoprotein I (anti-β_2GPI) (1). The aPL are a heterogeneous family of autoantibodies directed against phospholipid-binding plasma proteins, such as β_2GPI and prothrombin, or phospholipid–protein complexes, which are expressed on or bound to the surface of vascular endothelial cells, platelets, or monocytes (2). Recent evidence suggests that aPL antibodies alter the regulation of hemostasis and induce the activation of complement (3).

The serine protease thrombin is the main effector of the coagulation cascade. Thrombin generation, which is triggered by disruption of vascular integrity, allows plasma coagulation factors to come in contact with

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extravascular tissue factor (TF), the major initiator of coagulation in vivo. Thrombin converts circulating fibrinogen to fibrin monomer, which polymerizes to form fibrin, the fibrous matrix of blood clots. In addition, thrombin is a multifunctional protein that, aside from its function in hemostasis and thrombosis, has several cellular effects that link the coagulation system with the inflammatory response (4,5). Thrombin is able to provoke all of these responses in different cells through its protease-activated receptors (PARs). PARs are G protein–coupled receptors that carry their own ligands, which remain cryptic until unmasked by receptor cleavage. Proteases that are produced during vascular injury exert many of their cellular effects by cleaving and activating the PARs. Four main members of the PAR family have been identified to date: PAR-1, PAR-3, and PAR-4, which are thrombin receptors, and PAR-2, which is activated by trypsin, tryptase factor Xa, or factor VIIa (6,7).

Recent studies have suggested that signaling activities of TF are mainly mediated by PARs. TF is expressed at low levels on circulating monocytes and leukocyte-derived microparticles. These sources of intravascular TF can be tethered to activated platelets and endothelial cells and concentrated in this way at sites of injury or inflammation. This alters the local balance between activation and inhibition of the coagulation cascade and triggers thrombin production (8). Patients with primary APS have increased expression of TF on monocyte surfaces, increased messenger RNA (mRNA) for TF, and increased TF antigen and TF activity levels in peripheral blood mononuclear cells, where the source of TF is the monocyte (9–12). TF expression may contribute to thrombosis in patients with aPL. Moreover, molecular mechanisms associated with TF-induced expression by aPL activation in monocytes from APS patients have recently been delineated (13,14). It has also been demonstrated that in mice treated with aPL, complement activation, specifically C5, induces TF expression in neutrophils, which contributes to respiratory burst, trophoblast injury, and pregnancy loss (15). These data underline the relevance of TF as a prothrombotic and proinflammatory receptor that is involved in the development of the main clinical features of this autoimmune disease.

Clear evidence of relevant thrombin-independent, TF-mediated PAR signaling has been found in recent studies. These studies have shown that the TF–factor VII(a) complex activates PAR-2, and the product of initiation of coagulation, factor Xa, while still assembled in the transient ternary TF–factor VII(a)–factor Xa complex, signals through PAR-1 or PAR-2 (16,17).

PARs play important roles in normal physiology and disease. The major contribution of PAR family members to thrombin responses in platelets and endothelial cells has been well-established (18). It has recently been demonstrated that human peripheral monocytes and monocyte-derived macrophages and dendritic cells differentially express PARs. Human monocytes express mainly PAR-1 and less PAR-3. Differentiation of monocytes into macrophages induced by either macrophage colony-stimulating factor (M-CSF) or granulocyte–macrophage colony-stimulating factor (GM-CSF) elicits enhanced expression of PAR-1, PAR-2, and PAR-3 (19).

Considering the significance of monocytes for thrombosis in the setting of APS (2,3,12), we investigated the expression of PARs in these cells, as well as their correlation with the expression of other molecules relevant to the development of thrombosis in this autoimmune disorder. Our results provide the first demonstration of increased expression of PAR-1 and PAR-2 in monocytes from APS patients with thrombosis. We also demonstrated a correlation between PAR-2 levels with IgG aCL titers, as well as parallel behavior of TF and PAR-2 expression. Furthermore, in vitro studies suggested that there was direct cross-talk between TF and PAR-2, such that PAR-2 inhibition prevented the aCL-induced TF expression.

PATIENTS AND METHODS

Patients and controls. Sixty-two patients fulfilling the classification criteria for APS (1) were included after approval was obtained from the ethics committees. All patients signed informed consent forms. Patients were divided into 2 groups: group 1 consisted of 37 patients with previous thrombotic events, and group 2 consisted of 25 patients with recurrent pregnancy loss. We excluded all APS patients who had evidence of an underlying systemic rheumatic disease. As controls, we studied 20 patients with thrombosis but without APS (group 3) and 20 age- and sex-matched healthy donors (group 4). None of the healthy controls had a history of autoimmune disease, bleeding disorders, thrombosis, or pregnancy loss. All but 3 of the patients were studied at least 9 months after their latest thrombotic event or pregnancy loss. In 3 control patients in group 3, the blood sample was taken 4 months after the thrombotic event.

Groups 1 and 3 included only patients with objectively verified thrombotic events. No patients had protein C, protein S, or antithrombin deficiency. No patients were receiving treatment with any immunomodulatory agents, since they did not have any other underlying systemic autoimmune disease. All of the APS patients with thrombosis were receiving treatment with an oral anticoagulant (dicumarol). APS patients without thrombosis were receiving low-dose aspirin therapy (125 mg/day) or were receiving no treatment. Some of the control patients with thrombosis but without APS were taking oral anticoagulants, some were taking aspirin, and the rest were receiving no treatment.

Table 1. Characteristics of the patients with primary antiphospholipid syndrome (APS) and the controls

Variable	Primary APS patients		Controls	
	With thrombosis (n = 37)	Without thrombosis (n = 25)	Thrombosis without APS (n = 20)	Matched healthy donors (n = 20)
No. female/male	23/14	25/0	8/12	13/7
Age, years				
Median ± SD	44.4 ± 2.3	41.4 ± 2.3	46.8 ± 9.3	39.6 ± 4.3
Range	18–67	27–55	25–71	24–55
Thromboses, no. (%)				
Venous	12 (32)	0	9 (45)	0
Arterial	24 (65)	0	12 (60)	0
Recurrent	17 (46)	0	5 (25)	–
No. (%) with miscarriages	9 (24)	25 (100)	0	0
Anticardiolipin antibodies				
IgG isotype	22 (60)	16 (64)	Negative	Negative
IgM isotype	19 (51)	12 (48)	Negative	Negative
No. (%) with lupus anticoagulant	25 (68)	18 (72)	Negative	Negative

All patients were tested for the presence of aCL and LAC: IgG and IgM aCL were determined by enzyme-linked immunosorbent assay (ELISA) using a standardized commercial kit (Inova Diagnostics, San Diego, CA). The results were expressed in standard IgG phospholipid (GPL) or IgM phospholipid (MPL) units/ml. Values in the range of 15–20 GPL or MPL units/ml (inclusive) were considered negative. Values >20 GPL or MPL units/ml were considered positive, with the degrees of positivity defined as follows: 20–40 GPL or MPL units/ml = low-positive, 40–80 GPL or MPL units/ml = medium, and >80 GPL or MPL units/ml = high-positive results. Anti-β_2GPI antibody activity was evaluated by ELISA (using a kit from Inova Diagnostics), and the results were reported semiquantitatively in standard IgG anti-β_2GPI units (SGU). LAC was detected according to the guidelines recommended by the Standardisation Committee of the International Society on Thrombosis and Haemostasis (20). The characteristics of the patient and control groups are shown in Table 1.

Isolation of monocytes. Peripheral venous blood samples from patients and controls were collected into precooled sterile tubes containing 0.129*M* sodium citrate (1/9 [volume/volume]; Becton Dickinson Vacutainer System Europe, Meylan, France) as the anticoagulant and were centrifuged immediately at 500*g* for 10 minutes at 4°C to remove platelets. Isolation of monocytes from peripheral blood was performed by depletion of nonmonocytes by use of a commercially available kit (Monocyte Isolation Kit II; Miltenyi Biotec, Bergisch Gladbach, Germany), which enabled us to obtain monocytes without activation. Briefly, peripheral blood mononuclear cells, which were obtained by density-gradient centrifugation over Ficoll-Paque, were depleted of T cells, natural killer cells, B cells, dendritic cells, and basophils by indirect magnetic labeling using a cocktail of hapten-conjugated CD3, CD7, CD19, CD45RA, CD56, and anti-IgE antibodies and microbeads coupled to an antihapten monoclonal antibody (mAb). Magnetically labeled cells were removed by retention on a column in a magnetic field. The purity of the isolated monocytes was evaluated by staining aliquots of the cell fraction obtained with a fluorochrome-conjugated antibody against monocytes (fluorescein isothiocyanate [FITC]–conjugated anti-CD14) and then analyzing by flow cytometry. By this method, 86.7 ± 3% viable monocytic cells (mean ± SEM) were obtained.

RNA isolation and quantitative real-time reverse transcription–polymerase chain reaction (RT-PCR). Total cellular RNA from monocytic cells was extracted using TRI Reagent (Sigma, St. Louis, MO) according to the manufacturer's recommendations. The RNA concentration was determined spectrophotometrically at 260 nm and 280 nm, and its integrity was verified following separation by electrophoresis in a 0.8% agarose gel containing ethidium bromide. RNA samples were stored at –80°C until used in the experiments.

The expression levels of genes for TF, PARs 1–4, and GAPDH (as the housekeeping gene) were measured by quantitative real-time RT-PCR using a LightCycler Thermal Cycler system (Roche Diagnostics, Indianapolis, IN). The sequences of the primers used in the study and the theoretical size of the PCR products were as follows: for TF, 5′-CTACTGTTTCAGTGTTCAAGCAGTG-3′ (forward) and 5′-CAGTGCAATATAGCATTTGCAGTAGC-3′ (reverse), 283 bp; for PAR-1, 5′-ACCCGCAGAAGTCAGGAGA-3′ (forward) and 5′-GCCGCACAGACTGAAGCA-3′ (reverse), 98 bp; for PAR-2, 5′-GGCCGCCATCCTGCTAG-3′ (forward) and 5′-TGTGCCATCAACCTTACCAATAA-3′ (reverse), 63 bp; for PAR-3, 5′-GCAGCTGCTGGCCTCCT-3′ (forward) and 5′-AATGGGTAAGGTTGGCTTTGC-3′ (reverse), 55 bp; for PAR-4, 5′-GCTGCTGCATTACTCGGAC-3′ (forward) and 5′-ACGTAGGCACCATAGAGGTTG-3′ (reverse), 60 bp; and for GAPDH, 5′-TGATGACATCAAGAAGGTGGTGAAG-3′ (forward) and 5′-TCCTTGGAGGCCATGTAGGCCAT-3′ (reverse), 240 bp.

One-step RT-PCR was performed using a QuantiTect SYBR Green RT-PCR kit (Qiagen, Hilden, Germany) (14). Messenger RNA samples were analyzed in at least 3 similar RT-PCR procedures. Negative controls containing water instead of RNA were run to confirm that the samples were not cross-contaminated. Relative mRNA 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 that in controls.

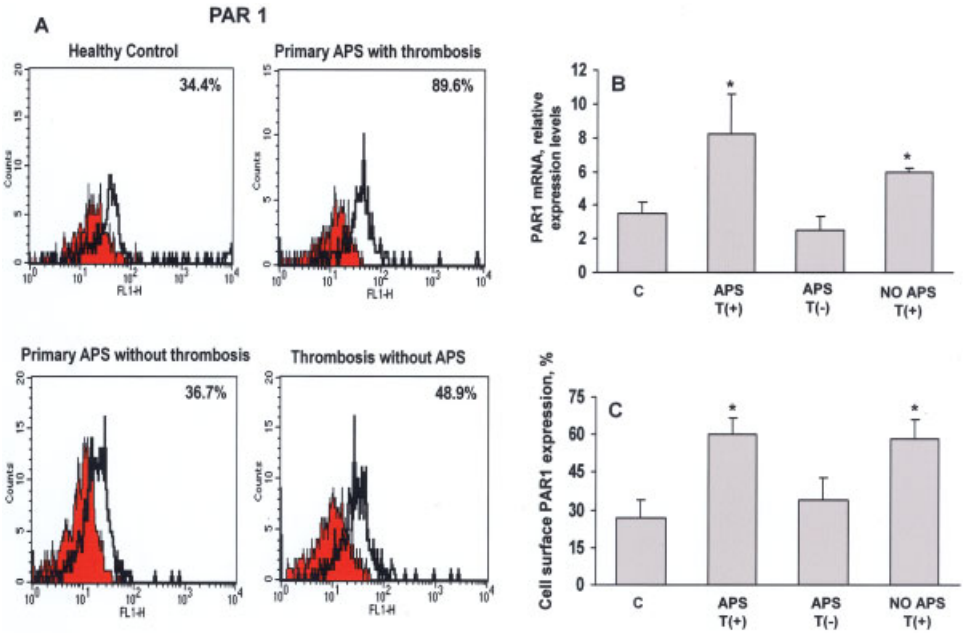

Figure 1. Expression of protease-activated receptor 1 (PAR-1) in purified monocytes from patients with antiphospholipid syndrome (APS). **A,** Detection by flow cytometric analysis of PAR-1 expression on monocytes from a representative healthy control subject, an APS patient with thrombosis, an APS patient without thrombosis, and a thrombosis patient without APS. Open histograms show specific staining for PAR-1, which is also indicated as a percentage (see Patients and Methods for details); closed histograms represent cells stained with isotype-matched control antibodies. **B** and **C,** Relative expression of mRNA for PAR-1 (**B**) and cell surface expression of PAR-1 protein (**C**) in monocytes from healthy controls, APS patients with thrombosis (T), APS patients without thrombosis, and thrombosis patients without APS. Values are the mean and SEM. $* = P < 0.05$ versus healthy controls and versus APS patients without thrombosis.

Flow cytometry. Cells were washed in phosphate buffered saline (PBS), incubated for 30 minutes at 4°C with PBS containing 20% heat-inactivated normal human serum, washed again, and then incubated for 30 minutes at 4°C with 15 n*M* concentrations of specific mAb to human TF (clone TF9-6B4, FITC-conjugated; American Diagnostica, Stamford, CT), to human PARs 1–4 (FITC-conjugated; Santa Cruz Biotechnology, Santa Cruz, CA), or to human CD14 (R-phycoerythrin [PE]–conjugated; Caltag, South San Francisco, CA). Flow cytometry analyses were performed on a FACScan (BD Biosciences, San Jose, CA). Control cells incubated with FITC- or PE-conjugated nonspecific antibodies obtained from the same manufacturers were used to set the threshold for the fluorescence parameter, such that the fraction of cells with positive fluorescence was <2.5% of the total cells. The percentage of TF, PARs 1–4, or CD14+ cells was determined from the fraction of cells in the sample incubated with specific antibodies that exceeded the threshold for the fluorescence signal intensity obtained with the control sample.

Purification of IgG and in vitro exposure of normal monocytes to aCL, lipopolysaccharide (LPS), and mAb against PAR-1 and PAR-2. IgG from the pooled sera of 7 patients with APS (characterized by high-titer aCL [>120 GPL units]) and a history of thrombosis, as well as from the pooled sera of 10 healthy subjects (as controls) was purified by protein G–Sepharose high-affinity chromatography (MabTrap kit) according to the recommendations of the manufacturer (Amersham Biosciences, Uppsala, Sweden). The absence or presence of endotoxin in IgG fractions was determined by *Limulus* amebocyte lysate assay (Amebo-lysate; ICN Biomedicals, Costa Mesa, CA). All IgG preparations tested negative in this assay. The anti-β_2GPI antibody activity of purified IgG was confirmed by ELISA and was reported semiquantitatively in SGU. The mean ± SD anti-β_2GPI antibody activities of the IgG aCL and control IgG were 93.2 ± 15.8 SGU and 6.8 ± 0.6 SGU, respectively. IgG from all patients selected for this study had moderate to high activity of anti-β_2GPI, which correlated with the level of IgG observed in these patients ($P = 0.0179$, $r = 0.898$).

For in vitro studies, monocyte populations purified from healthy donors were cultured with serum-free RPMI 1640 containing 2 m*M* L-glutamine, 100 units/ml of penicillin, 100 mg/ml of streptomycin, and 250 pg/ml of Fungizone (BioWhittaker/MA BioProducts, Walkersville, MD), at 37°C in a humidified atmosphere consisting of 5% CO_2. Purified normal monocytes (1.5×10^6/ml) were incubated for 6 hours at 37°C with purified APS patient IgG (200 μg/ml), normal human serum IgG, or LPS (1 μg/ml). To test the effect of blocking the active site of PAR-1 or PAR-2, monocytes were pretreated for 2 hours at 37°C with 1 μg/ml of anti–PAR-1 mAb (ATAP2) or anti–PAR-2 mAb (SAM11), then incubated for 6 hours with purified APS patient IgG or normal human serum IgG as described above.

Statistical analysis. All data are expressed as the mean ± SEM. Statistical analyses were performed using the SigmaStat software package (Jandel Scientific, Erkrath, Germany). Before comparing 2 data groups, a normality test and

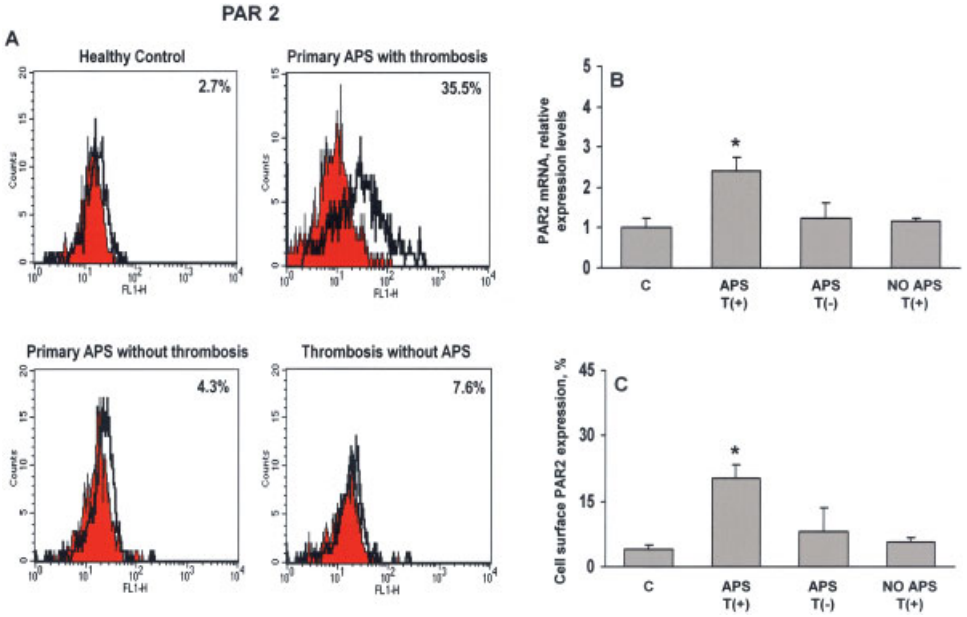

Figure 2. Expression of protease-activated receptor 2 (PAR-2) in purified monocytes from patients with antiphospholipid syndrome (APS). **A,** Detection by flow cytometric analysis of PAR-2 expression on monocytes from a representative healthy control subject, an APS patient with thrombosis, an APS patient without thrombosis, and a thrombosis patient without APS. Open histograms show specific staining for PAR-2, which is also indicated as a percentage (see Patients and Methods for details); closed histograms represent cells stained with isotype-matched control antibodies. **B** and **C,** Relative expression of mRNA for PAR-2 (**B**) and cell surface expression of PAR-2 protein (**C**) in monocytes from healthy controls, APS patients with thrombosis (T), APS patients without thrombosis, and thrombosis patients without APS. Values are the mean and SEM. $* = P < 0.05$ versus healthy controls, APS patients without thrombosis, and thrombosis patients without APS.

an equal variance test were performed. If data groups passed both tests, a comparison was made by a parametric test (Student's paired *t*-test). If the normality and/or equal variance test was violated, a comparison was made by a nonparametric test (Mann-Whitney rank sum test). Correlations were assessed by Spearman's rank order correlation. *P* values less than 0.05 were considered significant.

RESULTS

Titers of aCL antibodies and gene and protein TF expression in monocytes from APS patients. Anticardiolipin antibodies of IgG isotype were positive in 22 of the 37 patients with primary APS and thrombosis (mean ± SEM 54.6 ± 9.1 GPL units) and in 16 of the 25 patients with primary APS patients but without thrombosis (38.1 ± 5.2 GPL units). Anticardiolipin antibodies of IgM isotype were positive in 19 of the 37 primary APS patients with thrombosis (mean ± SEM 31.2 ± 8.3 MPL units) and in 12 of the 25 primary APS patients without thrombosis (22.7 ± 11.6 MPL units). LAC was positive in 43 of the 62 patients (69%).

TF mRNA levels were increased significantly in preparations of total RNA derived from uncultured monocytes obtained from 38 of the 62 APS patients (31 of 37 with thrombosis and 7 of 25 without thrombosis [61%]; mean ± SEM relative expression in all APS patients 4.62 ± 0.9), as compared with the control patients with thrombosis but without APS (1.72 ± 0.7; $P < 0.001$) and with the healthy controls (0.69 ± 0.09; $P < 0.001$). Subgroup analysis showed that the mean levels of TF mRNA were significantly higher in monocytes from patients with a history of thrombosis (5.73 ± 1.58) than in those from patients without thrombosis (1.86 ± 0.62; $P < 0.049$). Accordingly, cell surface–associated TF was detected in samples from 38 of the 62 APS patients, and the levels were significantly increased in patients with thrombosis (mean ± SEM 39 ± 3.7% positive cells) as compared with those in patients without thrombosis (24.3 ± 2.9%; $P < 0.001$), control patients with thrombosis but without APS (17.5 ± 0.9%; $P < 0.001$), and healthy control subjects (7.8 ± 2.4%; $P < 0.001$).

Expression of PARs 1–4 in monocytes from APS patients. Analysis of mRNA revealed that PAR-1 was the most abundant member of the PAR family identified in the monocytes of APS patients (mean ± SEM 10.22 ± 2.8; $P < 0.001$) (Figure 1B). Significantly increased

expression of PAR-2 was also observed in the APS patients (2.42 ± 0.42) as compared with the expression in the healthy control group ($P = 0.047$) (Figure 2B). PAR-3 expression (0.68 ± 0.20) was not significantly altered in comparison with the healthy controls, and PAR-4 expression was absent.

Similarly, the results of the flow cytometry analyses showed significantly higher levels of protein PAR-1 and PAR-2 levels in primary APS patients as compared with those in the healthy controls ($P = 0.001$ and $P = 0.045$, respectively) (Figures 1A and C and Figures 2A and C), suggesting that monocytes might constitute a relevant source of PARs 1 and 2 in primary APS patients.

Subgroup analysis showed that cell surface PAR-1 expression in APS patients with previous thrombosis (mean ± SEM 60.2 ± 6.3%) was significantly higher than that in APS patients without thrombosis (41.7 ± 11.2) and healthy controls (26.55 ± 7.5), but did not show any difference in comparison with patients with thrombosis but without APS (58.6 ± 7.6%) (Figures 1A and C). These data suggested that PAR-1 expression is increased in prothrombotic states, but it does not seem to be directly associated with APS. Cell surface PAR-2 was significantly higher in patients with APS and thrombosis (20.3 ± 3.1) as compared with all the other groups (8.2 ± 5.2% in APS patients without thrombosis, 5.7 ± 1.2 in control patients with thrombosis but without APS, and 4.1 ± 0.9 in healthy controls; $P < 0.05$ for each comparison) (Figures 2A and C).

Correlations among aCL, PARs, and TF levels. Spearman's relational statistic indicated a positive correlation between cell surface PAR-2 and PAR-1 expression levels among all APS patients analyzed ($r = 0.72$, $P = 0.0033$) (Figure 3A). In addition, there was a significant rank correlation between the expression levels of PAR-2 and TF ($r = 0.64$, $P = 0.0051$) (Figure 3C). Subgroup analysis further demonstrated a significant rank correlation between the expression levels of PAR-2 and TF ($r = 0.465$, $P = 0.0044$) in APS patients with thrombosis. We also found a positive correlation between the expression levels of PAR-2 and TF ($r = 0.95$, $P = 0.0018$) in APS patients without thrombosis. However, in the control patients with thrombosis but without APS, we found a significant rank correlation only between the expression levels of TF and PAR-1 ($r = 0.83$, $P = 0.0053$).

Levels of PAR-2 correlated with the titers of IgG aCL and with the activity of anti-β_2GPI antibodies, but only in the APS patients with thrombosis ($r = 0.366$, $P = 0.042$) (Figure 3B). We did not find any correlation among the PARs or TF levels and the IgM aCL titer or the positivity for LAC.

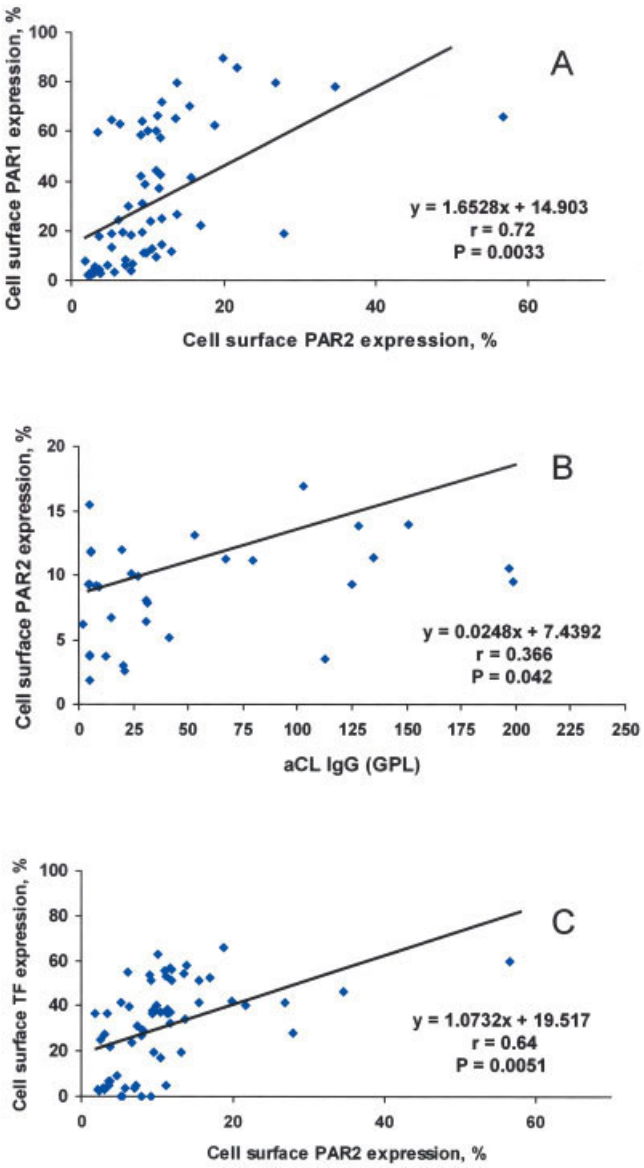

Figure 3. Correlations between protease-activated receptors (PARs) 1 and 2 and IgG anticardiolipin antibody (aCL) and tissue factor (TF) expression. Positive correlations were identified for cell surface expression of PAR-1 and PAR-2 (**A**), for cell surface expression of PAR-2 and the titer of IgG aCL (IgG phospholipid [GPL] units) (**B**), and for cell surface expression of TF and PAR-2 (**C**) in patients with antiphospholipid syndrome (APS). Correlations were assessed by Spearman's relational statistic, with significance set at $P < 0.05$. Data in **A** and **C** represent all study APS patients; data in **B** represent APS patients with thrombosis.

Up-regulation of monocyte PARs and TF expression induced by aCL. As shown in Figure 4, IgG from APS patients significantly increased cell surface expres-

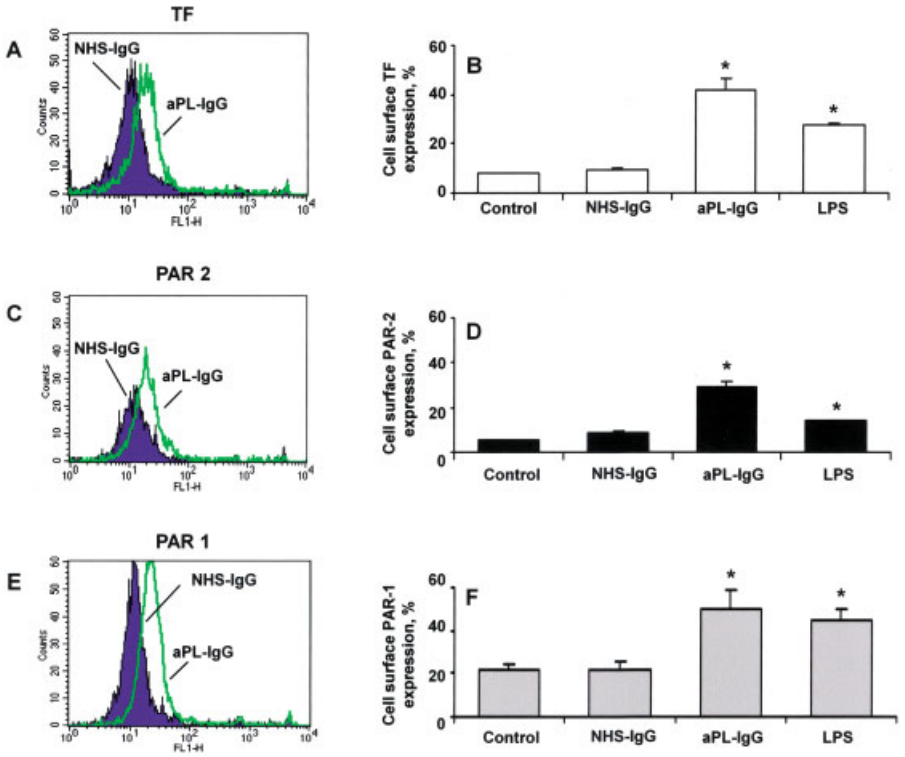

Figure 4. Expression of anticardiolipin antibody (aCL)–induced protease-activated receptors (PARs) and tissue factor (TF) in human monocytes. Normal human monocytes were incubated for 6 hours with purified aCL patient IgG (200 μg/ml), purified IgG from normal human serum (NHS), or lipopolysaccharide (LPS; 1 μg/ml). **A, C,** and **E,** Representative results from flow cytometric analyses of TF, PAR-1, and PAR-2. Results from 1 of 7 independent experiments are shown. **B, D,** and **F,** Cell surface levels of TF, PAR-2, and PAR-1. Values are the mean and SEM of 7 experiments. $* = P < 0.05$ versus untreated cells and versus normal human serum IgG–treated cells.

sion of monocyte TF, PAR-1, and PAR-2 as compared with IgG from the controls. These levels were increased to levels similar to those promoted by treatment with LPS.

Effect of inhibition of PARs on the aCL-induced expression of TF, PAR-1, and PAR-2. Inhibition of PAR-1 did not prevent the aCL-induced expression of TF in monocytes (Figure 5). However, treatment with the anti–PAR-2 mAb (SAM11) significantly abrogated the aCL-induced expression of TF. These data suggest that aCL-induced monocyte TF activation is mediated by the TF/factor VIIa/PAR-2 axis, and that cross-talk between the two molecules might be functional in these cells after treatment with aCL. On the other hand, the pretreatment of monocytes with specific mAb against PAR-1 or PAR-2 each prevented the aCL-induced activation of the other receptor, thus pointing to a direct association between both receptors in our cellular setting.

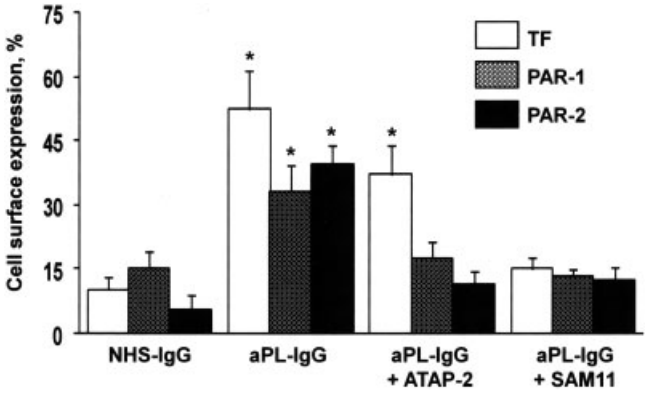

Figure 5. Effect of protease-activated receptors (PARs) inhibition on the antiphospholipid antibody (aPL; specifically, anticardiolipin antibody [aCL])–induced expression of tissue factor (TF), PAR-1, and PAR-2. Normal monocytes were pretreated for 2 hours at 37°C with 1 μg/ml of anti–PAR-1 (ATAP2) or anti–PAR-2 (SAM11) monoclonal antibody, incubated for 6 hours with purified aCL patient IgG (200 μg/ml) or purified IgG from normal human serum (NHS), and cell surface levels of TF, PAR-1, and PAR-2 were determined. Values are the mean and SEM of 4 experiments. $* = P < 0.05$ versus normal human serum IgG–treated cells.

DISCUSSION

Our data suggest that monocyte activation by aCL leads to up-regulation of the expression of PARs. In previous studies, we and other investigators have demonstrated the direct involvement of aCL-induced TF expression and activation of a tightly regulated intracellular cascade in the promotion of a prothrombotic state in patients with APS (13,14). Our observation that protease receptors 1 and 2 are also implicated in the process of monocyte activation induced by aCL further reinforces the idea of a more complex mechanism that is responsible for the development of thrombosis in APS.

In our studies on the thrombotic mechanism in APS, we have included only patients with primary APS. APS associated with systemic lupus erythematosus (SLE) occurs more frequently than primary APS and is very similar clinically (i.e., elevated levels of aPL and thrombotic events). The question of whether primary APS is a distinct clinical entity from APS associated with SLE is a subject of controversy. In fact, we have also found an overexpression of PARs in monocytes from patients with APS associated with SLE (López-Pedrera C, et al: unpublished observations), but the interpretation of these findings might be much more complex and not exclusively related to the effect of aCL in these patients. Mechanisms of inflammation are major contributors to the pathogenesis of SLE, but their role in APS is still not clear (21).

It is widely accepted that in addition to its role in coagulation, TF contributes to various pathophysiologic processes. Cell signaling induced by TF–factor VIIa (or TF–factor VIIa–factor Xa) or cell signaling induced by coagulation proteases generated by TF–factor VIIa might account for the altered cellular processes in APS (22). PAR-1 is a primary thrombin receptor. Thrombin activates PAR-1 to mediate its major proinflammatory effect, including the induction of interleukin-6 (IL-6), IL-8, monocyte chemotactic protein 1 (MCP-1), platelet-derived growth factor (PDGF), P-selectin, or vascular endothelial growth factor (VEGF). The action of PAR-1–dependent thrombin also enhances intracellular Ca^{2+} mobilization, ERK-1/2 phosphorylation, and activation of the transcription factor NF-κB (23). Similarly, PAR-2 mediates factor Xa signaling to induce the expression of IL-6, PDGF, or MCP-1, as well as ERK activation. It has also been shown that in TF-expressing cells, factor VIIa elicits VEGF and activates PAR-2 to enhance smooth muscle cell activation, MAPK phosphorylation, and Ca^{2+} mobilization.

Our data further suggest a direct association between both PARs since they are coexpressed and significantly increased in monocytes from APS patients with thrombosis. In support of this hypothesis, our in vitro studies demonstrated that the addition of specific mAb against either PAR-1 or PAR-2 significantly inhibited the induction of both receptors promoted by treatment with aCL. Thus, PAR-1 and PAR-2 might act together to provide redundant pathways by which to induce a prothrombotic/proinflammatory state. Moreover, it might be hypothesized that there is a direct involvement of PAR signaling in constitutive kinase activation and in the increased TF and VEGF expression already demonstrated in monocytes from patients with APS (17,24).

TF and PAR-2 are typically coexpressed only upon macrophage differentiation (19). Accordingly, in our study, increased monocyte PAR-2 expression correlated positively with the levels of both mRNA and cell surface TF expression in monocytes from APS patients with thrombosis. In vitro studies showed inhibition of aCL-induced TF expression after the addition of the specific PAR-2 inhibitor SAM11. However, inhibition of PAR-1 did not prevent monocyte TF expression after aCL treatment. Recent studies provided in vitro and in vivo evidence of a close reciprocal-signaling cross-talk between TF and PAR-2 (25–27). This close connection may indicate that TF-dependent signaling is tightly controlled in PAR-2–expressing cells. Thus, it might be suggested that PAR-2 is a key component that contributes to the regulatory control of TF initiation-phase signaling through PARs, as previously demonstrated in other cellular and pathologic settings (17). We found a positive correlation between the expression of PAR-2 and the levels of IgG aCL in the plasma of our APS patients. Our data further support the findings of a recent study showing that PAR-2 is up-regulated in neutrophils from mice treated with human IgG–containing aCL and that this receptor stimulation led to neutrophil activation, trophoblast injury, and fetal death (28). In conjunction with our results, the overall data might indicate the involvement of PAR-2 as a possible mediator of the aCL-induced fetal death and thrombosis in APS patients.

In conclusion, this study is the first to clearly show in vivo the increased expression of PAR-1 and PAR-2 in monocytes from patients with APS. Our in vitro findings indicated that both PARs were produced by monocytes when treated with aCL. Thus, these receptors might play a role as regulatory factors in aCL-mediated monocyte activation and TF expression and may therefore contribute to the proinflammatory–

prothrombotic phenotype observed in monocytes from APS patients. It is well-established that TF down-regulation and anticoagulation prevent and limit the progression of the coagulation cascade in the generation of proinflammatory coagulant signals, while PAR antagonists block the transmission of such signals. These approaches might be of significance in interrupting the coagulation/inflammation cycle and, thus, might be used as a novel therapy in APS patients with thrombosis.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. 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.

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