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Antiphospholipid Antibodies From Patients With the Antiphospholipid Syndrome Induce Monocyte Tissue Factor Expression Through the Simultaneous Activation of NF-κB/Rel Proteins Via the p38 Mitogen-Activated Protein Kinase Pathway, and of the MEK-1/ERK Pathway

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Objective. **Antiphospholipid syndrome (APS) is characterized by thrombosis and the presence of antiphospholipid antibodies (aPL). In patients with primary APS, expression of tissue factor (TF) on the surface of monocytes is increased, which may contribute to thrombosis in these patients. However, the intracellular mechanisms involved in aPL-mediated upregulation of TF on monocytic cells are not understood. This study was undertaken to investigate the intracellular signals induced by aPL that mediate TF activation in monocytes from APS patients.**

Methods. **We analyzed, both in vivo and in vitro, aPL interactions with proteins that have signaling functions, including mitogen-activated protein kinases (MAP kinases) and NF-κB/Rel proteins.**

Results. **In vivo studies demonstrated significantly higher levels of both TF messenger RNA and TF protein in monocytes from APS patients compared with controls. At the molecular level, increased proteolysis of IκBα and activation of NF-κB were observed. Constitutive activation of both p38 and ERK-1 MAP kinases was also found. Treatment of normal monocytes with aPL activated ERK-1 and p38 MAP kinases, as well as the IκB/NF-κB pathway, in a dose-dependent manner. NF-κB activation and IκBα degradation induced by aPL were inhibited by the NF-κB inhibitor SN50 and the p38 MAP kinase inhibitor SB203580, thus suggesting crosstalk between these pathways. However, the MEK-1/ERK inhibitor PD98059 did not affect aPL-induced NF-κB binding activity. TF expression induced by aPL was significantly inhibited by combined treatment with the 3 inhibitors.**

Conclusion. **Our results suggest that aPL induces TF expression in monocytes from APS patients by activating, simultaneously and independently, the phosphorylation of MEK-1/ERK proteins, and the p38 MAP kinase–dependent nuclear translocation and activation of NF-κB/Rel proteins.**

The antiphospholipid syndrome (APS) is characterized by thrombosis and/or pregnancy morbidity in the presence of antiphospholipid autoantibodies (aPL) (1). Among the many thrombogenic mechanisms proposed, it has been suggested that aPL can stimulate tissue factor (TF) expression by endothelial cells (ECs) and monocytes (2–5). Patients with primary APS have increased expression of TF on the monocyte surface, along with increased levels of TF messenger RNA (mRNA) and of TF antigen and activity levels in peripheral blood mono-

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nuclear cells, where the source of TF is the monocyte (6–8).

TF expression may contribute to thrombosis in aPL. This effect might ultimately depend on antibody engagement of PL-binding proteins on the monocyte and the EC surface, and the consequent induction of intracellular signals that ultimately will result in up-regulation of cell surface proteins such us TF. Various groups (9–11) have shown that aPL induces activation of NF-κB in ECs. NF-κB comprises a family of inducible transcription factors that serve as important regulators of the host immune and inflammatory response (12). In resting cells, NF-κB resides in the cytoplasm in an inactive form, bound to an inhibitory protein known as IκBα. Upon cellular activation by extracellular stimuli, IκBα is phosphorylated and proteolytically degraded or processed by proteasomes and other proteases. This proteolytic process activates NF-κB, which then translocates into the nucleus. NF-κB then binds to κB enhancer elements of target genes and induces transcription of a large number of genes (13).

A number of investigators have found that serum, plasma, purified total IgG, and anti–β_2-glycoprotein I (anti-β_2GPI) antibodies from APS patients enhance TF expression and procoagulant activity on normal monocytes (14). However, whether activation of NF-κB is required and what other intracellular mechanisms may be involved in aPL-mediated up-regulation of TF on monocytic cells are only partially understood.

NF-κB/Rel proteins regulate the inducible expression of many genes in activated monocytes and ECs that contain decameric κB and κB-like binding sites. NF-κB has been shown to be highly activated at sites of inflammation in diverse diseases, including rheumatoid arthritis, atherosclerosis, and asthma (15,16). However, there are no available data related to NF-κB activation in vivo in monocytes from patients with primary APS.

Depending on the nature of both the stimulus and the cell type, protein kinases are frequently activated, ultimately phosphorylating transcription factors and inducing new gene expression. Among these protein kinases, those belonging to the mitogen-activated protein kinase (MAP kinase) superfamily have received particular attention, because many of them translocate to the cell nucleus, where they regulate the activity of transcription factors that have important roles in cellular processes such as the regulation of cell proliferation, differentiation, and death (17).

To date, at least 3 MAP kinase cascades have been identified. They are known as the ERK/MAP kinase pathway, the JNK/SAPK pathway, and the p38 MAP kinase pathway. The ERK pathway is activated by many growth factors and hormones and is involved in mediating cell proliferation, transformation, and differentiation and TF expression. In contrast, the JNK and p38 cascades have been implicated in cell death and protective responses triggered by cytokines and environmental stress (18).

The p38 MAP kinase is activated by a wide spectrum of stimuli, and several pieces of evidence suggest that p38 plays a key role in regulating inflammatory responses. Some experimental data also implicate a role for p38 in regulating NF-κB activity (19,20). Both NF-κB and MAP kinases, in particular p38 MAP kinase, are activated by an often very similar pattern of agents, including proinflammatory cytokines, lipopolysaccharide (LPS), phorbol esters, phosphatase inhibitors, and ionizing and ultraviolet irradiation. The considerable overlap of stimulating conditions hints at the possibility that the 2 pathways may utilize common signaling molecules.

In this study, we investigated the intracellular signals induced by aPL that mediate the activation of TF in monocytes from APS patients, through analysis of the effects of aPL on proteins that have signaling functions, such as MAP kinases and NF-κB/Rel proteins. Our findings indicate that aPL induces TF gene and protein expression in monocytes from APS patients by simultaneously and independently activating the phosphorylation of p38 MAP kinases and nuclear translocation and activation of NF-κB/Rel proteins, and the phosphorylation of MEK-1/ERK proteins.

PATIENTS AND METHODS

Patients. Forty-four patients (38 nonpregnant women and 6 men; mean age 41 years [range 23–60]) fulfilling the Sapporo criteria for APS (21) were enrolled in this study after ethics committee approval was obtained. All patients provided written informed consent. Thirty-one of 44 patients (70%) had an objectively verified history of thrombosis (arterial in 22 [50%] and venous in 9 [20%]), and 13 (30%) had only recurrent pregnancy loss. All patients were studied at least 9 months after their latest thrombotic event or pregnancy loss. We excluded APS patients who had evidence of an underlying systemic disease or antibodies against double-stranded DNA or extractable nuclear antigen. All patients were tested for the presence of anticardiolipin antibodies (aCL), anti-β_2GPI activity, and lupus anticoagulant (LAC) as previously described (6). Briefly, anti-β_2GPI activity and IgG and IgM aCL were determined by enzyme-linked immunosorbent assay (ELISA) using a commercial kit (Cheshire Diagnostic, Chester, UK). For the detection of LAC, activated partial thromboplastin time, kaolin clotting time, and dilute Russell's viper venom time were measured according to the guidelines recommended

by the Subcommittee for Standardisation of the International Society on Thrombosis and Haemostasis (22). IgG and IgM aCL were positive in 32 and 17 patients, respectively, and LAC in 30. In addition, a direct correlation between the titers of IgG aCL and those of anti-β_2GPI activity was demonstrated.

Ten of the 44 patients (23%) had high blood pressure (defined as systolic pressure >140 mm Hg and diastolic pressure >80 mm Hg). Thirteen (29.5%) had hypercholesterolemia (defined as a total cholesterol level >5.0 mg/dl).

As controls, we studied 10 age- and sex-matched healthy subjects (6 nonpregnant women and 4 men; mean age 38 years [range 26–49]). None of the controls had a history of autoimmune disease, bleeding disorders, thrombosis, or pregnancy loss.

Monocyte isolation. Peripheral venous blood samples from patients and controls were collected in sterile precooled tubes containing 0.129*M* sodium citrate (1/9 [volume/volume]; Becton Dickinson Vacutainer System Europe, Meylan, France) as the anticoagulant and centrifuged immediately at 500*g* for 10 minutes at 4°C to remove platelets. Isolation of monocytes from peripheral blood was performed by magnetic depletion of non-monocytes, using a commercially available kit (Miltenyi Biotec, Bergisch Gladbach, Germany). 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 analysis by flow cytometry. By this method, 86.7 ± 3% viable monocytic cells (mean ± SEM) were obtained.

Purification of IgG and determination of antibodies. IgG from 7 patients with APS (all with a history of thrombosis) and from the pooled sera of 10 healthy subjects (as controls) was purified by protein G–Sepharose high-affinity chromatography (MAbTrapTM 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 (Amebolysate; ICN Biomedicals, Costa Mesa, CA).

Anti-β_2GPI antibody activity of purified IgG was confirmed by ELISA and reported semiquantitatively in standard IgG anti-β_2GPI units (SGU). IgG and aCL were determined as described above, and titers were reported in IgG phospholipid units (GPL).

In vitro exposure of normal monocytes to aPL. For in vitro studies, monocyte populations 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. Purified normal monocytes (1.5×10^6/ml) were incubated with various doses of purified APS patient IgG (100, 50, and 25 μg/ml) or normal human serum (NHS) IgG for 6 hours at 37°C. In some experiments, cells were pretreated for 30 minutes with SN50 (50 μg/ml), SB203580 (1 μM), or PD98059 (10 μM) (all from EMD Biosciences, San Diego, CA). Then purified APS IgG was added and cells were further incubated for 6 hours. The inhibitors used were dissolved in ethanol or dimethyl sulfoxide, so that the final concentration of solvent (0.1%) did not affect the growth or viability of the cells. Control cells were treated with the same concentration of diluent.

RNA isolation and quantitative real-time reverse transcriptase–polymerase chain reaction (RT-PCR). Total cellular RNA from monocytic cells was extracted using TRI-reagent according to the recommendations of the manufacturer (Sigma, St. Louis, MO). The expression levels of TF gene, and of GAPDH as a housekeeping gene, were measured by quantitative real-time RT-PCR using the LightCycler thermal cycler system (Roche Diagnostics, Indianapolis, IN). The sequence of primers used for study and the theoretical size of PCR products were as follows: TF (283 bp) forward primer CTACTGTTTCAGTGTTCAAGCAGTGA, reverse primer CAGTGCAATATAGCATTTGCAGTAGC; GAPDH (240 bp) forward primer TGATGACATCAAGAAGGTGGTGAAG, reverse primer TCCTTGGAGGCCATGTAGGCCAT. RT-PCR was performed in one step using the QuantiTect SYBR Green RT-PCR kit according to the protocol of the manufacturer (Qiagen, Hilden, Germany). Briefly, 100 ng of total RNA was placed into a 20-μl reaction volume containing 150 n*M* of each primer, 10 μl of SYBR Green PCR master mix, and 0.2 μl of reverse transcriptase. Complementary DNA (cDNA) was synthesized at 50°C for 20 minutes, followed by a step of HotStar *Taq* DNA polymerase activation, Omniscript and Sensiscript reverse transcriptase deactivation, and template cDNA denaturation at 95°C for 15 minutes, and 50 cycles of denaturation at 94°C for 15 seconds, annealing at 60°C for 30 seconds, and extension at 68°C for 40 seconds. The temperature transition rate was set at 20°C/second. Detection of the fluorescent product was performed at the end of the extension at 68°C. To confirm amplification specificity, the PCR products were subjected to a melting curve analysis from 65°C to 90°C.

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. Quantification of relative expression was determined by standard curve method according to the instructions of the manufacturer (Qiagen), and the target amount was normalized to the GAPDH gene. TF mRNA expression was considered positive in patient samples in which the value was more than 2-fold higher than the values obtained in control samples.

Flow cytometric analysis for detection of TF expression. Flow cytometric analysis was performed as previously described (6), using a FACScan (BD Biosciences, San Jose, CA) and specific monoclonal antibodies to human TF (clone TF9-6B4; FITC-conjugated) (American Diagnostica, Greenwich, CT) or to human CD14 (phycoerythrin [PE]–conjugated) (Caltag, South San Francisco, CA). Control cells incubated with FITC- or PE-conjugated nonspecific antibodies from the same manufacturer were used to set the threshold for the fluorescence parameter, such that the fraction of cells with positive fluorescence was <2.5% of total cells. The percentage of TF- or CD14-positive cells was determined from the fraction of cells in the sample incubated with specific antibodies that had a fluorescence signal intensity exceeding the threshold obtained with the control sample. TF expression was considered positive if it exceeded the control threshold by >15%. CD14 was considered positive if fluorescence was evident in >10% of cells.

Immunocytochemistry and ELISA for tumor necrosis factor α (TNFα) and interleukin-1β (IL-1β). The cytokines TNFα and IL-1β were measured using commercially available ELISA kits (Factor-Test hTNF-alpha and Intertest-1X, respectively; Genzyme, Boston, MA).

Western blotting and electrophoretic mobility shift assay (EMSA). Cytoplasmic and nuclear extracts were prepared according to standard protocols (23) and used for Western blotting and EMSA, respectively. Cytoplasmic extracts (50 μg) were resolved by sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis and then transferred to nitrocellulose membranes. IκBα, phosphorylated and nonphosphorylated p38, and ERK-1/2 MAP kinase protein levels were determined by Western blotting using monoclonal or polyclonal anti-IκBα, anti–phospho-p38 MAP kinase, anti–p38 MAP kinase, anti–phospho-ERK-1/2, anti–ERK-1/2, and anti-actin antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) (24). Visualization of immune complexes was performed using horseradish peroxidase–conjugated secondary antibodies, and the Luminol reagent detection system (Santa Cruz Biotechnology). Protein levels were quantified using the image analysis software Intelligent Quantifier, version 2.1.1 (Bio Image, Ann Arbor, MI). Results were calculated in terms of integrated optical density (IOD) and expressed in arbitrary units (AU).

Sequence-specific DNA binding activity of NF-κB protein was assayed by EMSA. The consensus sequence for NF-κB (5′-AGTTGAGGGGACTTTCCCAGGC-3′; Santa Cruz Biotechnology) was labeled with γ^{32}P-ATP using T4 polynucleotide kinase. The reaction mixtures containing 10 μg of nuclear extracts, 1 μg poly(dIdC), and the labeled probe were incubated in binding buffer (10 m*M* Tris HCl [pH 7.5], 50 m*M* NaCl, 0.5 m*M* EDTA, 1 m*M* $MgCl_2$, 4% glycerol) for 30 minutes at room temperature. DNA–protein complexes were separated from unbound probe by electrophoresis on a 6% polyacrylamide gel with a running buffer of 0.5× TBE (50 m*M* Tris HCl [pH 7.5], 50 m*M* borate, 1 m*M* EDTA). Complexes formed were identified by autoradiography of the dried gels. For antibody interference studies, incubation mixtures containing the corresponding specific antibody (anti-p65 or anti-p50; Santa Cruz Biotechnology) were preincubated for 15 minutes at room temperature. The labeled probe was then added for a further 30 minutes and electrophoresis was carried out on 4% polyacrylamide gels.

Statistical analysis. Data are expressed as the mean ± SEM. Statistical analysis was carried out using the software package SigmaStat (Jandel, Erkrat, Germany). Comparisons were made by parametric testing with Student's paired *t*-test. If the normality and/or equal variance test was violated, a comparison was made by nonparametric testing (Mann-Whitney test). Correlations were assessed by Pearson product-moment correlation. *P* values less than 0.05 were considered significant.

RESULTS

Gene and protein TF expression in monocytes from APS patients. Forty-four patients with primary APS and 10 healthy controls were included in the study. TF mRNA levels were found to be significantly increased in total RNA preparations from uncultured monocytes from 29 of 44 patients (66%) (23 of 31 with thrombosis and 6 of 13 without thrombosis) ($P < 0.001$ compared with healthy controls). Moreover, mean levels of TF mRNA were significantly higher in monocytes from patients with a history of thrombosis (mean ± SEM relative expression level 3.71 ± 0.2) than in those from patients without thrombosis (1.68 ± 0.3) ($P < 0.035$) and from healthy controls (0.78 ± 0.1) ($P < 0.001$).

Cell surface–associated TF was detected in samples from 29 of the 44 APS patients, and its levels in patients with thrombosis were significantly increased (49.2 ± 3.9% positive cells) compared with the levels observed in patients without thrombosis (21.6 ± 3.6%) and in healthy controls (5.6 ± 1.3%) (both $P < 0.001$). Cell TF expression was significantly higher in the group of 10 patients who were hypertensive than in the 34 who were normotensive ($P < 0.013$ by Fisher's exact test). There was no statistically significant difference in cell TF expression between the group of 13 patients with high cholesterol levels and the 31 with normal cholesterol levels ($P = 0.321$ by Fisher's exact test). Of note, 5 of the 13 hypercholesterolemic patients were receiving statins.

Pearson's relational statistic indicated that the level of TF correlated positively with the titer of IgG aCL ($r = 0.708$, $P = 0.0098$) as well as with level of anti-β_2GPI activity ($r = 0.644$, $P = 0.03$), but not with IgM aCL or LAC levels.

TNFα and IL-1β expression. There were no significant differences between patients and controls in the serum levels of TNFα (mean ± SEM 30.9 ± 1.6 pg/ml versus 21.3 ± 0.68 pg/ml, respectively) and IL-1β (0.97 ± 0.06 versus 0.78 ± 0.08 pg/ml, respectively).

DNA binding NF-κB activity in monocytes from APS patients. To analyze the molecular mechanism triggered by aPL in monocytes from patients with primary APS and to study the significance of NF-κB activation in vivo, NF-κB binding activity was determined, by EMSA, in nuclear extracts of monocytes from patients with APS. Monocytes from 26 of the 44 APS patients showed both increased proteolysis of the cytoplasmic inhibitory protein IκBα and activation of NF-κB proteins p65 and p50 compared with controls. Representative EMSAs from APS patients and healthy controls are depicted in Figure 1. Among the 26 patients exhibiting increased NF-κB binding activity, 17 had a history of thrombosis, 13 of whom additionally showed increased TF expression. The proteins binding to the NF-κB consensus motif were characterized as members

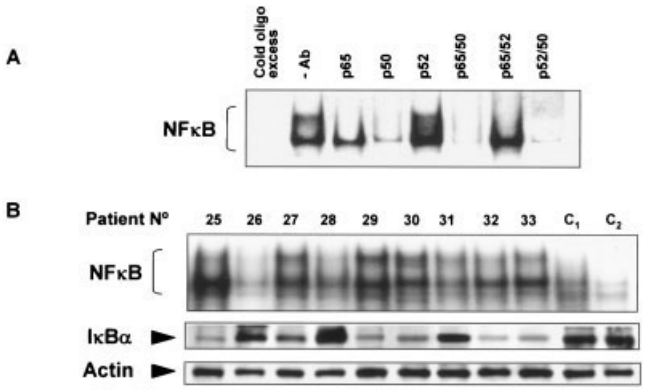

Figure 1. NF-κB binding activity in monocytes from patients with antiphospholipid syndrome (APS). **A,** The proteins binding to the NF-κB consensus motif were characterized as members of the NF-κB family based on the interaction with antibodies (Ab) directed against proteins of the NF-κB family. **B,** Representative electrophoretic mobility shift assay results in samples from 9 APS patients and 2 healthy controls (C), performed using ^{32}P–NF-κB consensus oligonucleotide, as described in Patients and Methods. Cytoplasmic extracts were subjected to Western blot analysis using anti-IκBα and antiactin antibodies.

of the NF-κB family based on the interaction with antibodies directed against proteins of the NF-κB family (Figure 1A).

Activation of p38 and ERK-1/2 MAP kinases in monocytes from APS patients. Levels of phosphorylated forms of p38 MAP kinases, as measured by densitometry, were significantly higher in APS patients (mean ± SEM 3,999.7 ± 226.7 AU of IOD) compared with controls (1,674.2 ± 275.3 AU of IOD) ($P < 0.001$). In addition, there was a significant negative correlation between levels of p38 MAP kinase and IκBα ($P = 0.018$) (Figure 2B). Thus, increased levels of p38 MAP kinase correlated with diminished levels of cytosolic IκBα expression. Phosphorylated forms of ERK-1, but not ERK-2, MAP kinases were also found to be increased in APS patients (2,919.9 ± 320.3 AU of IOD) compared with controls (1,492.8 ± 398.4 AU of IOD) ($P = 0.041$), but no significant correlation with IκBα levels was observed. In all cases a direct relationship between significantly elevated TF expression and activation of ERK-1 and/or p38 MAP kinase and/or NF-κB on the same sample could be established. Representative Western blots showing phosphorylated and nonphosphorylated forms of both MAP kinases from APS patients and healthy controls are depicted in Figure 2A.

Intracellular pathways induced by aPL: characterization of IgG aPL. Given the evidence that NF-κB is activated in monocytes from APS patients, and that

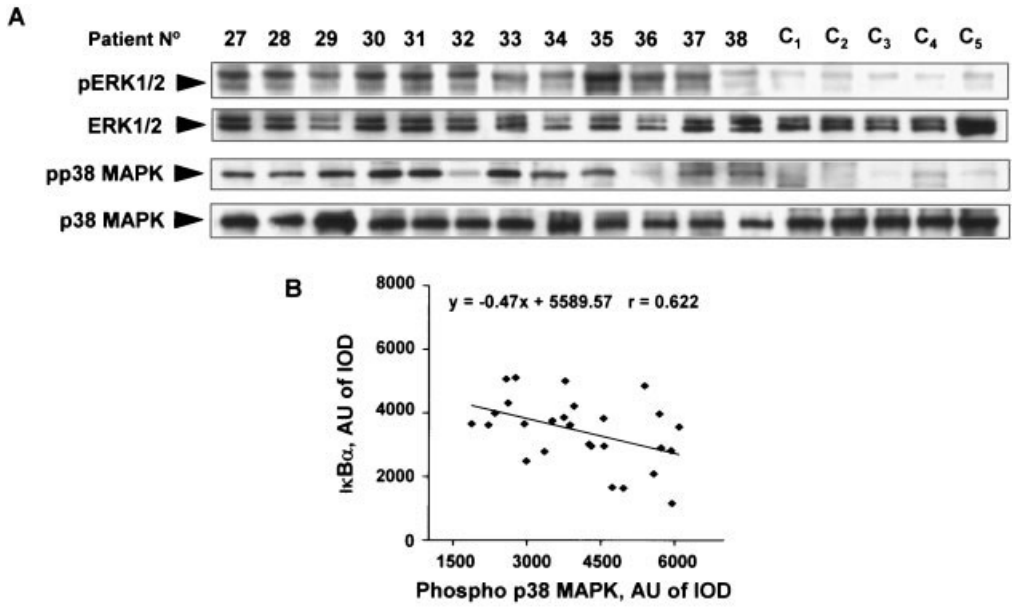

Figure 2. Activation of pERK and p38 mitogen-activated protein kinases (MAPKs) in monocytes from patients with antiphospholipid syndrome (APS). **A,** Representative Western blotting results in 12 APS patients and 5 healthy controls (C). pp38 MAPK = phosphorylated p38 MAPK. **B,** Negative correlation between IκBα and phosphorylated p38 MAPK expression levels, as assessed by Pearson product-moment correlation. AU = arbitrary units; IOD = integrated optical density.

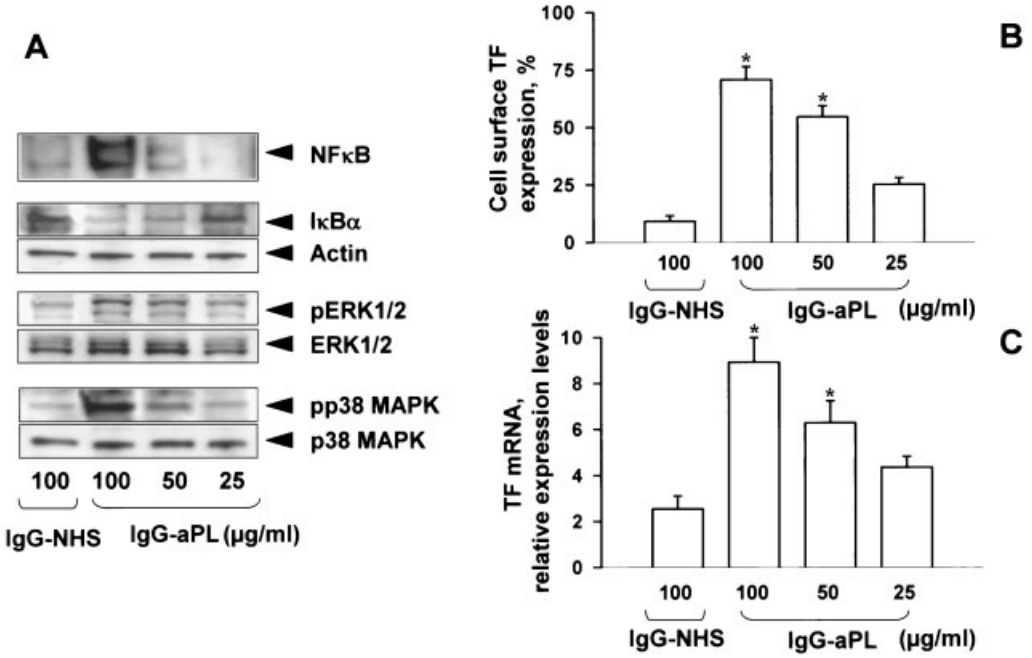

Figure 3. Activation of intracellular pathways by antiphospholipid syndrome (APS) patient IgG and dose dependence of APS IgG–induced tissue factor (TF) expression. **A,** Representative electrophoretic mobility shift assay and Western blotting results from 4 separate experiments showing, respectively, NF-κB binding activity and IκBα, pERK-1, and p38 MAP kinase (MAPK) expression after treatment with different doses of IgG antiphospholipid antibody (aPL) or IgG from normal human serum (NHS) at maximal concentration. pp38 MAPK = phosphorylated p38 MAPK. **B** and **C**, TF mRNA relative expression levels and cell surface TF expression in monocytes treated with various doses of IgG aPL. Values are the mean and SEM from 4 experiments. $* = P < 0.05$ versus healthy controls.

ERK and p38 MAP kinases are also activated in these cells, we sought to investigate whether these intracellular pathways are activated by aPL, and to what extent they are involved in the TF overexpression observed in monocytes from patients with APS. Pooled sera from 7 patients with APS were used to prepare IgG and applied in all in vitro experiments. 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 ± SEM anti-β_2GPI antibody activities of the IgG aPL and control IgG were 87.2 ± 19.3 SGU and 5.3 ± 0.6 SGU, respectively. All patient IgG preparations selected for this study had moderate-to-high anti-β_2GPI activity, which correlated with the level of IgG observed in these patients ($r = 0.904$, $P = 0.0352$). All IgG preparations were negative for LPS by *Limulus* amebocyte lysate assay.

Induction of NF-κB, pERK, and p38 MAP kinase activation by aPL. Antiphospholipid antibody treatment activated ERK-1/2 and p38 MAP kinases in normal human monocytic cells, as well as the classic inflammatory IκB/NF-κB pathway, in a dose-dependent manner (Figure 3A). To further characterize these effects, the 3 pathways were analyzed after combined treatment with specific inhibitors (SN50, SB203580, and PD98059). EMSA studies demonstrated that NF-κB activation induced by aPL was inhibited by both the specific NF-κB inhibitor SN50 (25) and the p38 MAP kinase inhibitor SB203580 (26), thus suggesting involvement of the p38 MAP kinase pathway in NF-κB activation in monocytes from APS patients. In contrast, PD98059, the specific MEK-1/ERK inhibitor (27), did not affect aPL-induced nuclear translocation of NF-κB proteins (Figure 4).

Involvement of NF-κB, p38, and MEK-1/ERK-1 activation in the aPL-induced up-regulation of monocyte TF expression. IgG from 7 APS patients significantly increased monocyte TF expression at both the mRNA and protein levels compared with IgG from controls, in a dose-dependent manner (Figures 3B and C). TF values reached a maximum after treatment with 100 μg/ml of IgG aCL (mean ± SEM TF mRNA expression 8.9 ± 1.1 relative expression levels in treated

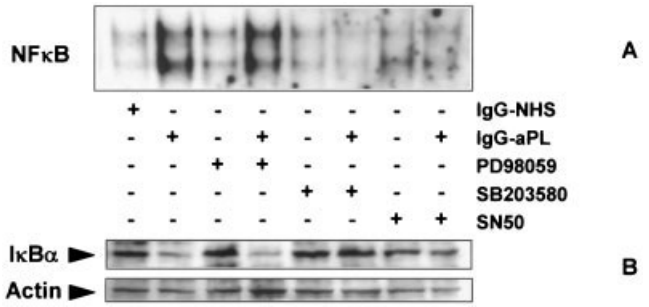

Figure 4. NF-κB binding activity and IκBα expression in monocytes exposed to combined treatments with APS patient IgG and specific cell signaling inhibitors. Normal purified monocytes were pretreated with PD98059 (10 μM), SN50 (50 μg/ml), or SB203580 (1 μM) for 30 minutes, followed by IgG aPL exposure for 6 hours. Cytosolic and nuclear lysates were prepared, and electrophoretic mobility shift assay (EMSA) and Western blotting were performed. **A,** Representative results of EMSA performed using ^{32}P–NF-κB consensus oligonucleotide. **B,** Results of Western blotting performed using anti-IκBα and antiactin antibodies. Results shown are representative of 3 independent experiments with similar results. See Figure 3 for other definitions.

cells versus 2.5 ± 0.6 relative expression levels in control cells; cell surface TF expression 70.8 ± 5.6% in treated cells versus 9.1 ± 2.5% in control cells) (both $P < 0.001$). The expression of TF induced by aPL on normal monocytic cells was also examined in the presence and in the absence of the 3 inhibitors. As shown in Figure 5, TF expression at both the mRNA and protein levels was significantly reduced by the 3 inhibitors, PD98059 being the most effective. These data indicate involvement of activation of NF-κB, p38, and MEK-1/ERK-1 in aPL-induced up-regulation of TF.

DISCUSSION

The data obtained in this study strongly suggest that circulating monocytes from patients with APS are subjected to up-regulated TF expression as a consequence of aPL activation. This is, to our knowledge, the first study in which the aPL-induced intracellular signals that mediate the activation of TF in monocytes in APS have been further delineated and characterized using in vivo and in vitro analyses.

Transcription factors and cell signaling molecules, such as NF-κB and MAP kinases, are present in almost all blood cell types. Therefore, to specifically quantify and evaluate the expression and activation of such molecules in monocytic cells, we used an isolating system for this cell type. This system enabled us to obtain monocytes in the absence of activation, which allowed ascertainment of whether circulating monocytes really show an activated state and increased TF expression in APS. To avoid methodologic variations, the culture conditions were strictly controlled through both the use of minimally adherent culture plates and the study of cell response in controls after culture under comparable conditions.

Our results primarily demonstrate that IgG fractions from APS patients induce TF expression on normal blood monocytes. The effect of APS patient IgG was dose dependent, and the magnitude of TF expression was comparable with that observed in monocytes from APS patients. TF expression was not related to the presence of the IgM aCL isotype.

The signal transduction mechanisms leading to the increased expression of TF in monocytes from APS patients are largely unknown. Recent data indicate the involvement of activation of NF-κB and of p38 MEK-1 in aPL-mediated up-regulation of TF in endothelial cells (9,20). TF has NF-κB–responsive elements in the gene promoter/enhancer (28,29). However, the pathway leading to the nuclear translocation of NF-κB in monocytes from APS patients remains to be elucidated. In the present study we observed that monocytes from these patients showed increases in both proteolysis of the cytoplasmic inhibitory protein IκBα and nuclear translocation and activation of the NF-κB proteins p65 and p50, compared with controls. Moreover, redistribution of NF-κB from the cytoplasm to the nucleus was accompanied by increased expression of TF.

Activation of the NF-κB transcription factor family by nuclear translocation of cytoplasmic complexes plays a central role in inflammation through its ability to induce transcription of proinflammatory genes. In APS, aPL-induced NF-κB activation leads to up-regulation of gene transcription of adhesion molecules on ECs and to initiation of various signal transduction pathways. In that regard, Dunoyer-Geindre et al (10) have demonstrated that NF-κB is an essential intermediary in the activation of ECs by anti-β_2GPI. Moreover, cytokines such as TNFα and IL-1β are known to enhance the expression of TF in ECs and monocytes. The signaling pathways of these cytokines are quite different and very complex, but converge at the transcription factor NF-κB (30). In the present study, we analyzed serum levels of TNFα and IL-1β, the 2 cytokines that mainly influence TF production. There were no differences between patients and controls in the levels of these cytokines, suggesting that aPL are directly responsible for the induction of TF expression.

As in ECs, other signal transduction pathways

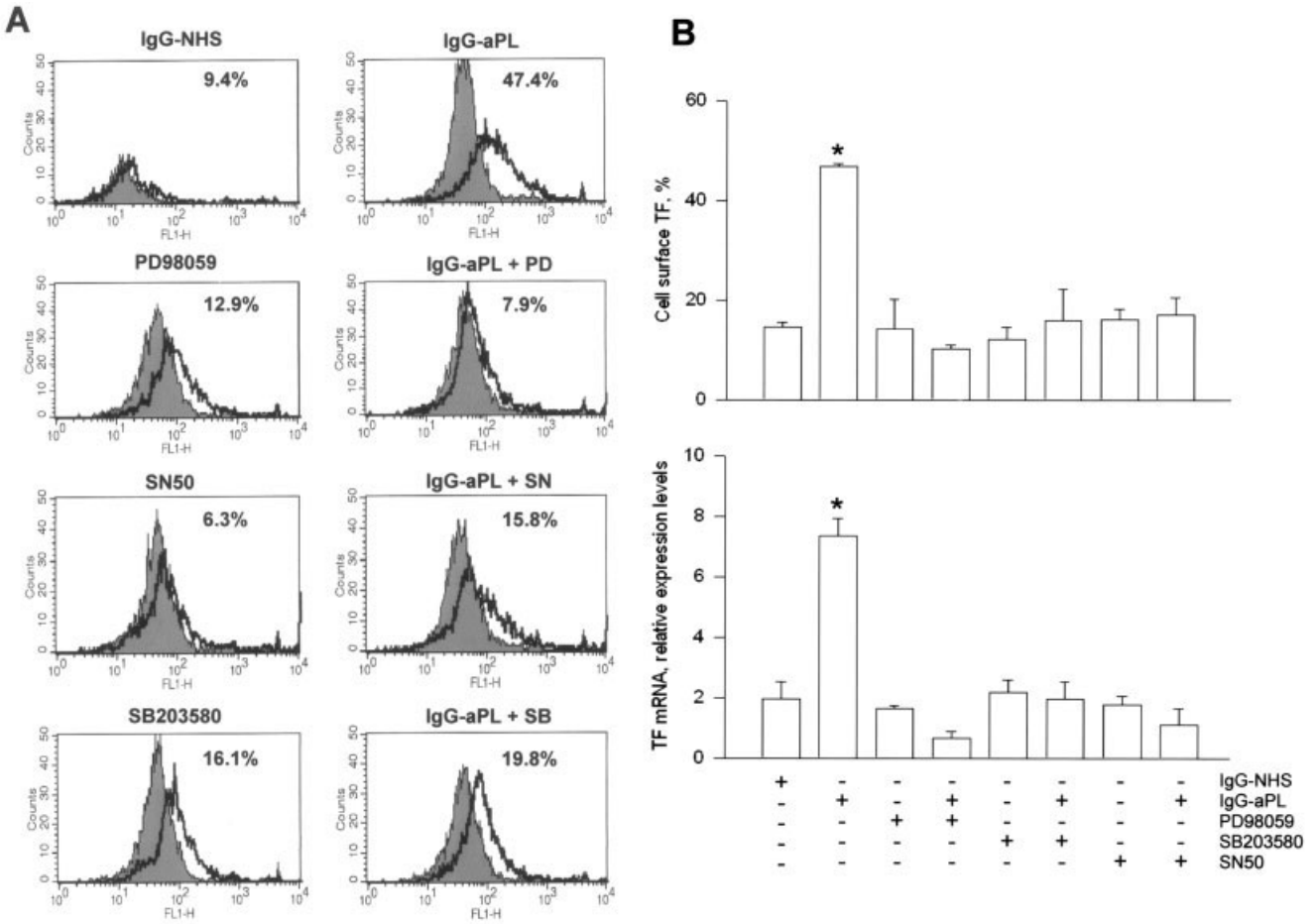

Figure 5. Intracellular pathways involved in the IgG aPL–induced monocyte TF expression. Normal monocytes were pretreated with PD98059 (PD; 10 μM), SN50 (SN; 50 μg/ml), or SB203580 (SB; 1 μM) for 30 minutes, followed by IgG aPL exposure (100 μg/ml) for 6 hours. TF mRNA relative expression levels and cell surface TF expression were then measured as described in Patients and Methods. **A,** Detection of cell surface TF on monocytes in a representative flow cytometry experiment. **B,** TF mRNA relative expression levels and cell surface TF expression on monocytes treated as described above. Values are the mean and SEM from 3 experiments. $* = P < 0.05$ versus healthy controls. See Figure 3 for other definitions.

may be turned on in monocytes, leading to NF-κB activation, and this would lead to the expression of cell surface receptors and to activation of monocytes upon exposure to aPL. Several lines of evidence suggest that MAP kinases can participate in the regulation of NF-κB transcriptional activity. Moreover, it has been demonstrated that the p38 pathway is involved in the activation of NF-κB in the cytoplasm as well as in modulation of its transactivating potential in the nucleus (19).

We found increased levels of the phosphorylated form of p38 MAP kinase in APS patients compared with controls, with a significant inverse correlation with diminished levels of cytosolic IκBα expression. In addition, when we analyzed the effect of SB203580, a highly specific inhibitor of p38 MAP kinase, we observed that the drug prevented the activation of NF-κB DNA binding activity induced by aPL treatment, as measured by EMSA. Thus, our data suggest that p38 MAP kinases are implicated in NF-κB activation in monocytes from patients with APS.

Activation of p38 MAP kinase is of interest, since this kinase is important in both inflammation and coagulation, making it an attractive candidate as a possible mediator of TF effects in pathologic conditions (31). In addition, the p38 MAP kinase pathway has been implicated in the regulation of TF expression in monocytes, ECs, and smooth muscle cells (32–36). Moreover, in many immunologic and oncologic disorders, activation

of p38 MAP kinase coincides with activation of the Raf/MEK/ERK signal transduction cascade (37–39). Activated MEK/ERK pathways specifically phosphorylate and transactivate transcription factors (such as Elk-1), which then regulate the expression of genes such as TF (40). In the present study, phosphorylated forms of ERK-1 MAP kinases were also found to be increased both in vivo in monocytes from APS patients, and in vitro after aPL treatment. Furthermore, analysis of the functional consequences of the pharmacologic disruption of these pathways, using selective inhibitors of NF-κB, MEK-1/ERK, and p38 MAP kinase activation, showed that the 3 inhibitors significantly reduced aPL-induced TF expression. Thus, the 3 pathways seem to be involved in the TF expression induced in monocytes by aPL.

However, while p38 MAP kinase and NF-κB pathways appear to be directly related, the MEK/ERK pathway seems to act through an independent mechanism. In vivo studies showed no significant correlation between ERK-1 and IκBα levels, although an inverse correlation was observed with p38 MAP kinase. Additionally, the MEK-1 inhibitor PD98059 strongly inhibited aPL induction of TF expression but did not affect the induced nuclear translocation of NF-κB proteins. Taken together, the above results suggest that the significant TF expression observed in monocytes from APS patients may be the consequence of the simultaneous activation of 2 main alternative and independent pathways, which are directly associated with the regulation of this receptor.

Our data provide the first in vivo demonstration that NF-κB, p38 MAP kinase, and ERK-1 play an important role in the expression of TF by monocytes from patients with primary APS. These results are consistent with the recent findings by Vega-Ostertag et al (41) suggesting that aPL induce activation of p38 MAP kinase in platelets, and those by Bohgaki et al (20) demonstrating p38 MAP kinase phosphorylation and NF-κB activation in a monocytic cell line in response to aPL/β_2GPI treatment. However, a major discrepancy between the findings of those studies and our present results is that in neither the platelets nor the monocytic cell line analyzed was activation of the MEK/ERK-1 kinase pathway observed. If a key role for ERK-1 in the regulation of monocyte TF expression is assumed, then its participation is not relevant in platelets, where minimal or no expression of TF is observed (42). This might explain the lack of activation of this kinase in platelets after aPL exposure. The absence of ERK pathway activation in the study by Bohgaki et al might be the

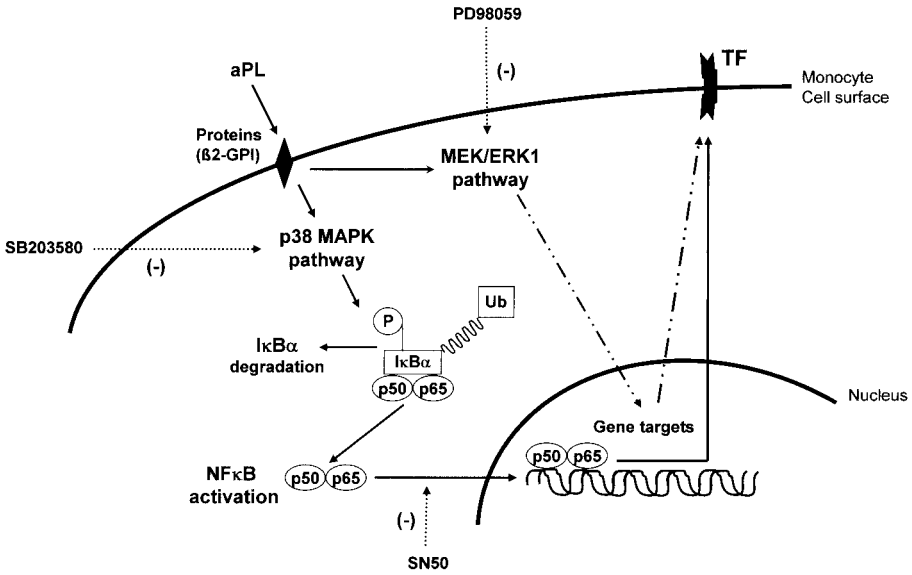

Figure 6. Intracellular signaling pathways activated by antiphospholipid antibodies (aPL) in monocytes, and the effects of specific inhibitors and their involvement in tissue factor (TF) expression. PD98059 is a specific inhibitor of the MEK1/ERK pathway; SB203580 is a specific inhibitor of the p38 MAP kinase pathway; SN50 is a specific inhibitor of NF-κB translocation. (−) = inhibitory effect; β_2GPI = β_2-glycoprotein I; P = phosphorus; Ub = ubiquitin.

result of methodologic differences in a study using a monocytic cell line.

Our data are, however, consistent with the results of a study of ECs performed by Pierangeli and Harris (43), thus suggesting that the activation of both ECs and monocytes by aPL results in activation of TF through the use of the same intracellular pathways, and supporting the notion that both cell types make a relevant contribution to the development of thrombosis in APS. The well-documented interaction between the 2 cell types at different levels further supports this premise.

Figure 6 illustrates our working hypothesis of the mechanisms involved in TF activation mediated by aPL in monocytes from patients with APS. Some aspects remain to be confirmed at the molecular level, e.g., that aPL might induce TF gene and protein expression in monocytes from APS patients by simultaneously and independently activating the phosphorylation of MEK-1/ERK proteins and the phosphorylation of p38 MAP kinases and subsequent nuclear translocation and activation of NF-κB/Rel proteins.

Theoretically, inhibition of monocyte TF expression is an attractive avenue for development of treatments to prevent thrombosis in APS and other hypercoagulability conditions. Understanding the intracellular mechanism(s) of aPL-mediated monocyte activation may help to establish new therapeutic approaches, such as selective inhibition of the mitogen-activated protein kinases, to reverse the prothrombotic state in APS.

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