Essential role of the p38 mitogen-activated protein kinase pathway in tissue factor gene expression mediated by the phosphatidylserine-dependent antiprothrombin antibody

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Abstract

Objective. The aim of this study was to investigate the effects of phosphatidylserine-dependent antiprothrombin antibody (aPS/PT) on the expression of tissue factor (TF) and the signal transduction pathway in procoagulant cells.

Methods. Peripheral blood mononuclear cells (PBMCs) from a healthy donor, murine monocyte RAW264.7 cells and human umbilical vein endothelial cells (HUVECs) were treated with either IgG fractions obtained from APS patients who were positive for aPS/PT or a murine monoclonal aPS/PT antibody, 231D, in the presence of prothrombin. The levels of TF mRNA were measured using real-time PCR. TF function, as measured by procoagulant activity, was determined with a clotting assay. 231D binding on the surface of treated cells was determined by flow cytometric analysis. Screening for phosphorylation of intracellular signalling proteins was conducted using an array assay. Phosphorylation of p38 MAPK was quantitatively analysed with ELISA, and SB203580 was used as a specific inhibitor of p38 MAPK. Specific siRNA for p38 MAPK was used for the knockdown assay.

Results. The IgG fractions from APS patients and 231D induced TF mRNA overexpression and shortening of coagulation time in cells in the presence of prothrombin. The 231D moiety induced phosphorylation of p38 MAPK after binding to the cell surface of RAW264.7 cells. SB203580 or p38 siRNA significantly hampered TF overexpression.

Conclusion. Expression of TF in procoagulant cells was induced by aPS/PT via p38MAPK phosphorylation. This phenomenon may be correlated with the thrombogenicity of APS.

Key words: antiphospholipid syndrome, antiprothrombin antibody, tissue factor, p38 MAPK, procoagulant cell activation.

Introduction

APS is a clinical condition characterized by recurrent thrombotic events and/or pregnancy morbidity associated with the persistence of aPLs. aPLs are a large and heterogeneous group of circulating immunoglobulins that appear either idiopathically or in a wide range of infectious or autoimmune diseases [1].

Traditionally aPLs are classified as aCLs, anti-beta-2-glycoprotein I (aβ2GPI) antibodies or LA. Both aCL and aβ2GPI are detected by ELISA, and both target the complex of β2GPI and anionic phospholipids. These antibodies are designated β2GPI-dependent anticardiolipin antibodies (aCL/β2GPI) [2]. LA is detected by functional coagulation tests that require a careful and sequential series of examinations, and LA activities are indicative of the existence of heterogeneous antibodies, including aCL/β2GPI.
Evidence has shown that some LA activities depend on antibodies against prothrombin, which was first proposed as a possible cofactor for LA in 1959 [3]. The pathogenicity of aPT was reported from various institutes [4, 5]. Haj-Yahia et al. [8] reported that aPT obtained from mouse immunized with human prothrombin showed pathogenicity in an ex vivo model. However, association between antiprothrombin and clinical manifestation of APS is still a subject of controversy [7].

We showed that antibodies against the phosphatidylserine–prothrombin complex (aPS/PT), rather than antibodies against prothrombin alone, are closely associated with APS and LA [8], and their targeted antigen is a complex of anionic phospholipid and its binding protein, an analogue of the cardiolipin–β2GPI complex. The sensitivity and specificity of aPS/PT for the diagnosis of APS have been assessed in a population with a variety of autoimmune disorders. It is now recognized that aPS/PT may have diagnostic potential, and they have been proposed as a candidate marker of APS and as an alternative test for LA [9–12].

In contrast to the clinical observation of a strong link between aPS/PT and thrombosis, only a few studies have demonstrated the thrombogenicity of aPS/PT. We have established a monoclonal aPT, designated 231D, which specifically binds to phosphatidylserine–prothrombin complex (PS/PT) and possesses strong LA activity [13]. The concentration-dependent LA activity of the monoclonal aPS/PT and the epitope overlap reasonably represent the characteristics of autoimmune aPS/PT.

Tissue factor (TF) is the initiator of the extrinsic coagulation pathway, and we previously reported its upregulation in APS patients [14, 15]. Further, the results of our previous study and those of other studies demonstrated that monoclonal aCL/β2GPI binds directly to procoagulant cells such as monocytes and endothelial cells (ECs), and that this binding mediates cell dysregulation, which may induce the clinical manifestations of APS [16–19]. When procoagulant cells are exposed to aCL/β2GPI in the presence of β2GPI, they produce thrombophilic molecules, particularly TF or adhesion molecules concomitant with activation of the p38 mitogen-activated protein kinase (MAPK) pathway [20–23]. Considering the analogy in the immunological aspects and clinical impact between aCL/β2GPI and aPS/PT, these two populations of antibodies are likely to share in the pathophysiology of APS.

In this study we investigated the effects of aPS/PT on procoagulant cells by performing in vitro assays with purified IgG fractions obtained from the sera of patients with APS who were positive for aPS/PT and negative for aCL/β2GPI, and with the monoclonal aPS/PT antibody, 231D.

Materials and methods

Monoclonal and autoimmune aPTs

Two murine monoclonal aPTs, 231D and 51A6, were previously established and characterized [13]. Briefly, the monoclonal aPS/PT antibody 231D was established as follows. BALB/c mice were intraperitoneally immunized with human prothrombin emulsified with complete or incomplete Freund’s adjuvant. Spleen cells were fused with P3U1 mouse myeloma cells, and cells producing antibodies against PS/PT complex were screened using an aPS/PT ELISA, and the monoclonal antibody was sequentially purified by protein G-Sepharose affinity chromatography. 51A6, the monoclonal antibody directed against prothrombin, was established in the same manner as 231D with the exception of the immunogen used, prethrombin-1, which is a fragment of prothrombin lacking the phospholipid-binding site (Gla domain).

Both monoclonals bind strongly to the PS/PT complex, but not to phosphatidylserine alone; however, 231D has stronger binding to the PS/PT complex than 51A6. 51A6 binds to prothrombin coated on both irradiated and non-irradiated ELISA plates (antiprothrombin-alone [aPT-A] activity [24]); however, 231D shows little binding to prothrombin regardless of the plate type. 231D-spiked plasma has strong LA activity; 51A6-spiked plasma also has LA activity, but it is weaker. Binding of purified IgG from aPS/PT-positive patients with APS to the PS/PT complex is partially inhibited by 231D, but not by 51A6. Therefore 231D has characteristics common to autoimmune aPS/PT. In contrast, 51A6 binding to prothrombin was not affected by the presence of phosphatidylserine, which is far different from the characteristics of the aPTs found in patients with APS.

IgG fractions were obtained from plasma samples of five APS patients with high titres of IgG aPS/PT in the absence of IgG aCL and aβ2GPI using protein G-Sepharose affinity chromatography (MAbTrap-TMII, Pharmacia). The patients included three females with a mean age of 46 (range 36–72) years, disease duration of 3–7 years and one to four past thrombotic events. IgG fractions from patients were pooled as the IgG aPS/PT fraction and frozen until use. Purified IgG fractions from plasma of three healthy individuals were prepared in the same fashion.

The study was performed in accordance with the Declaration of Helsinki and the principles of good clinical practice. Approval was obtained from the local ethics committee (Institutional Review Board of Hokkaido University Hospital), and informed consent was obtained from all subjects.

Cell isolation and preparation

Venous blood was collected from healthy donors into heparinized tubes. Peripheral blood mononuclear cells (PBMCs) were isolated by gradient centrifugation (Ficoll-Paque plus, GE Healthcare, Chalfont St Giles, Buckinghamshire, UK). The cells were then washed with Rosewell Park Memorial Institute (RPMI)-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal calf serum (GIBCO BRL, Paisley, UK) containing penicillin and streptomycin, followed by centrifugation once at 400 g for 5 min at room temperature, and twice for 5 min at 4 °C. The cells were then resuspended in RPMI-1640 and counted using the trypan blue dye exclusion method. The murine monocyte...
cell line RAW264.7 (American Type Culture Collection number TIB-71) was maintained in an atmosphere of 5% CO₂ at 37°C in DMEM (GIBCO BRL) supplemented with 10% heat-inactivated FCS containing penicillin and streptomycin. Human umbilical vein endothelial cells (HUVECs) (Kurabo, Tokyo, Japan) were maintained under 5% CO₂ at 37°C in HuMedia EB-2 (Kurabo).

Procoagulant cell treatment
Prothrombin, monoclonal aPS/PT (231D) and mouse IgG were added to PBMCs or RAW264.7 cells at a concentration of 10 μg/ml and to HUVECs at a concentration of 15 μg/ml. The IgG aPS/PT fraction (500 μg/ml) or control IgG fraction (500 μg/ml) was added to the cells. Lipopolysaccharide (LPS) was used as positive control at a concentration of 100 ng/ml. The Ca²⁺ concentration in each sample was adjusted to 2.5 mM, which was sufficient to facilitate the binding of prothrombin to phosphatidylserine. The cells were treated for 5 h for TF mRNA determination, for 15 min for p38 MAPK phosphorylation and for 12 h for clotting assay.

Flow cytometry assay with IIF staining
To observe the binding of monoclonal antibody to the cell surface, a flow cytometry assay with IIF staining was performed. Mouse monoclonal antibodies and control IgG were added to RAW264.7 with or without prothrombin. Cells were washed and collected after 4 h of incubation. Diluted FITC-conjugated AffiniPure donkey anti-mouse IgG antibody (Sigma-Aldrich Co.) was added to the cell suspension and then analysed with a flow cytometry (FACS) analyser.

RNA isolation and quantitative TaqMan real-time PCR
Total RNA was isolated from the cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and an RNeasy Mini kit (Qiagen GmbH, Hilden, Germany) and stored at −80°C until use.

Complementary DNA (cDNA) was generated using the SuperScript II first-strand cDNA pre-amplification system (Gibco BRL, Rockville, MD, USA) according to the random primer protocol provided by the manufacturer. The induction of mRNA was measured by real-time PCR using TaqMan Universal PCR Master Mix and gene-specific sets of Assay-on-Demand Gene Expression probes (Applied Biosystems, Foster City, CA, USA) with the ABI PRISM 7000 Sequence Detection System (Applied Biosystems).

Messenger RNA samples were analysed in at least three similar real-time PCR procedures. Negative controls containing water instead of RNA were simultaneously run to rule out cross-contamination. Relative expression was quantified by the ΔΔCt method and normalized to GAPDH.

Clotting assay
To evaluate the procoagulant activity of cells, the clotting time of PBMCs was measured using an automated STA-R coagulation analyzer (Diagnostica Stago, Asnière, France). After three washes in Tris-buffered saline (TBS) containing 0.05% Tween 20 (Sigma-Aldrich Co.) and 5 mM CaCl₂ (TBS-Tween-Ca), 2 ml of normal human plasma was added to 2 ml of cell fluid (1 × 10⁶ cells/ml). The reduction in the clotting time compared with the normal control sample was interpreted as increased coagulation function and was attributed to the expression of TF.

Detection of intracellular signal protein phosphorylation
For parallel determination of the relative phosphorylation levels of intracellular signal proteins, particularly MAPKs and other serine/threonine kinases, an array assay was performed using the Human Proteome Profiler Array kit (R&D Systems, Minneapolis, MN, USA) following the standard procedure provided by the manufacturer. Briefly, concentrated PBMC lysates obtained from normal healthy controls were adjusted according to the manufacturer’s instructions following exposure to the stimulators [231D (10 μg/ml) with and without prothrombin (10 μg/ml)] for 15 min. The lysates were added to the array and exposed to X-rays for 5 min.

Quantitative analysis of serine-threonine kinase phosphorylation by cellular activation ELISA
Quantitative analysis of intracellular signal phosphorylation in RAW264.7 mouse monocytes was performed using a Cellular Activation of Signaling ELISA (CASE) kit (SABiosciences Corporation, Frederick, MD, USA) following the standard method provided by the manufacturer. The phosphorylation of p38 MAPK, c-Jun N-terminal kinase (JNK), extracellular regulated kinase (ERK1/2) and Akt (protein kinase B) was carried out as follows. Briefly, experimentally treated cells were seeded in 96-well plates and fixed with paraformaldehyde. Two primary antibodies, one that recognizes phosphorylated serine-threonine kinases and another that recognizes serine-threonine kinases regardless of phosphorylation were used to detect the relative amount of phosphorylated serine-threonine kinases, which was assayed by measuring the optical density (OD) on an ELISA plate reader. The OD was measured at 450 nm and normalized to the cell number (OD₅₄₀). Then the OD ratio (OD₄₅₀/OD₅₄₀) of phospho-serine-threonine kinase-specific antibody (OD phospho-kinases) was normalized to the pan-serine-threonine kinase-specific antibody OD ratio (OD pan-kinases) under the same experimental conditions, indicating the relative extent of serine-threonine kinase phosphorylation (OD phosphokinases/OD pan-kinases). Finally, to determine the relative extent of target protein phosphorylation, the OD phosphokinases/OD pan-kinases ratio of each sample was compared with unstimulated samples to calculate the relative amount of serine-threonine kinase phosphorylation.

RNA interference
RNA interference was carried out with Accell small interfering RNA (siRNA; Dharmacon, Lafayette, CO, USA), pre-designed pools of four oligonucleotides, using the Accell siRNA delivery protocol following the manufacturer’s
instructions. Briefly, 8 h after plating, PBMC from healthy controls (5 × 10^5 cells/well) were transfected with 1 μM p38 MAPK-α (MAPK 14) Accell siRNA or Accell non-targeting siRNA in 100 μl Accell siRNA delivery media (Dharmacon). Cells were incubated at 37°C 72 h before assessment of RNAi knockdown effect.

Proteins
Fatty acid-free BSA was obtained from Sigma-Aldrich. LPS was removed from the antibody preparation by using DetoxiGel (Pierce, Rockford, IL, USA), and its absence was confirmed using the Limulus amebocyte lysate assay (Limulus ES-II Single Test Wako; Wako, Osaka, Japan). Human prothrombin was obtained from Enzyme Research (South Bend, IN, USA).

Statistical analysis
Means of the various treated and control groups were compared by Student’s unpaired t-test. SPSS II for Windows (SPSS Japan Inc., Tokyo, Japan) was used for all calculations.

Results
Upregulation of TF mRNA expression in PBMCs and RAW264.7 cells treated with IgG from APS patients’ plasma and monoclonal aPS/PT
Immunoglobulin G isolated from APS patients’ plasma and LPS significantly increased the expression of TF mRNA in PBMCs. In contrast, IgG from healthy controls did not increase the expression of TF mRNA in PBMCs (Fig. 1A).

231D in the presence of prothrombin significantly increased the expression of TF mRNA in PBMCs and in RAW264.7 cells. However, 231D in the absence of prothrombin or control IgG with prothrombin did not increase TF mRNA expression (Fig. 1B and C).

Procoagulant activity of PBMCs treated with aPS/PT
TF function in aPS/PT-treated cells, measured by procoagulant activity, was analysed using a clotting assay. The clotting time of the cell fluid from PBMCs treated with IgG isolated from APS patients’ plasma in the presence of prothrombin was significantly reduced. In contrast, the coagulation time of cell fluid treated with APS patients’ IgG alone or IgG from healthy controls with prothrombin was not reduced (Fig. 2A). In addition, the clotting time of the cell fluid from PBMCs treated with 231D in the presence of prothrombin was significantly reduced. The coagulation time of cell fluid treated with 231D alone or with control IgG and prothrombin was not reduced (Fig. 2B).

Monoclonal aPS/PT binding to the cell surface of RAW264.7 cells was detected by a flow cytometric assay with IIF staining
RAW264.7 were treated with monoclonal aPTs (51A6 and 231D) or control mouse IgG at 37°C under 5% CO₂ for 4 h. In the presence of prothrombin, 75.9% of 231D-treated cells bound to antibody, while only 41.4% of 51A6-treated cells and 0.5% of control IgG-treated cells bound to antibody. In the absence of prothrombin, cells treated with antibodies showed almost no binding to the 231D, 51A6 and control IgG antibodies (3.8%, 0.1% and 0.3%, respectively) (Fig. 3).

Intracellular signal protein phosphorylation in PBMCs treated with monoclonal aPS/PT
Results of the array assay showed phosphorylation of p38 (p38α) in PBMCs treated with 231D in the presence of prothrombin. However, no p38 phosphorylation was detected in cells treated with 231D in the absence of prothrombin. Phosphorylation of other serine/threonine kinases or other MAPK family proteins was also not detected, therefore p38 was presumed to be the major signal protein involved in monocyte activation by aPS/PT.

Quantitative analysis of intracellular signal phosphorylation in RAW264.7 cells treated with monoclonal aPS/PT
Based on the results of the array assay, serine-threonine kinases including p38 phosphorylation was quantitatively analysed using an ELISA CASE kit. In the presence of prothrombin, 231D significantly increased the relative amount of p38 phosphorylation compared with the untreated control up to 1.7-fold. There was no increase in the amount of relative p38 phosphorylation with 231D in the absence of prothrombin, or with control mouse IgG plus prothrombin (Fig. 4A). The relative amount of phosphorylation in other serine-threonine kinases such as JNK, ERK1/2 and Akt were not detected (Fig. 4B–D).

Effect of p38 MAPK inhibitor on PBMCs TF expression induced by monoclonal aPS/PT treatment
To elucidate the role of p38 MAPK in TF mRNA expression, we investigated the effect of a p38 MAPK inhibitor on cells treated with monoclonal aPS/PT. The p38-specific inhibitor SB203580 significantly reduced TF mRNA over-expression in 231D-treated PBMCs (Fig. 5A) and RAW264.7 cells compared with the untreated control (Fig. 5B). However, its inactive analogue SB202474 did not affect TF mRNA expression. Addition of SB203580 to 231D-treated cells decreased TF mRNA expression 80–90%.

Effect of siRNA reagents on PBMC TF expression induced by monoclonal aPS/PT treatment
The effect of p38 siRNA on PBMC TF mRNA expression induced by 231D treatment was investigated as indicated. The expression of TF mRNA on 231D-treated PBMCs was significantly offset by pre-treatment of p38 siRNA. In contrast, pre-treatment of control siRNA did not affect TF mRNA expression on 231D-treated PBMCs (Fig. 6).

Upregulation of TF mRNA expression and adhesion molecules in HUVECs induced by monoclonal aPS/PT
The expression of TF mRNA was significantly upregulated in HUVECs treated with 231D in the presence of
prothrombin (PT + 231D vs baseline, 231D alone and PT + control IgG: 2.5 ± 0.7 vs 1, 1.2 ± 0.3 and 1.31 ± 0.24; all, P < 0.005). The p38-specific inhibitor SB203580 significantly reduced TF mRNA overexpression in 231D-treated HUVECs [SB203580 (+) vs SB203580 (−): 1.4 ± 0.2 vs 2.5 ± 0.7; P < 0.005]; however, its inactive analogue SB202474 did not affect TF mRNA overexpression.

The expression levels of vascular cell adhesion molecule-1 (VCAM-1), platelet-endothelial cell adhesion molecule-1 (PCAM-1) and endothelin-1 mRNA were significantly upregulated 2- to 3-fold in HUVECs treated with 231D in the presence of prothrombin. However, in the absence of prothrombin, 231D did not affect the expression of these adhesion molecules (PT + 231D vs baseline, 231D alone and PT + control IgG; VCAM-1: 2.1 ± 0.6 vs 1, 1.4 ± 0.1 and 1.1 ± 0.2; PCAM-1: 2.8 ± 0.3 vs 1, 1.3 ± 0.0 and 1.8 ± 0.3; selectin: 2.0 ± 0.4 vs 1, 1.3 ± 0.2 and 1.1 ± 0.4; all, P < 0.01).

**Discussion**

In this study we showed that IgG fractions and monoclonal aPS/PT induced TF in procoagulant cells. Monoclonal aPS/PT bound to monocytes using prothrombin as a co-factor for binding. Further, we demonstrated that treatment by aPS/PT induces the phosphorylation of p38 MAPK in these procoagulant cells. The coagulation process in vivo is complicated and various cells or molecules other than monocytes or endothelial cells are involved. Obviously there are certain limitations in discussing aPS/PT-induced thrombosis from the current results. However, our results showed that TF, the key protein in the coagulation pathway, is overproduced by its main sources in the circulation, monocytes and endothelial cells. Upregulation of blood-borne TF indicates increased procoagulant activity that is considered one of the most important characteristics of aPL-induced thrombosis.
PBMCs obtained from normal healthy donors were exposed to the substances described below for 5 h. The cells were washed and then added to normal healthy plasma, and coagulation time was measured. The reduction in coagulation time was calculated by subtracting the coagulation time of each treated sample from that of unstimulated cells. The bars represent the mean ± S.E. of three independent experiments. *P < 0.005. (A) PBMCs treated with IgG fractions from patients positive for aPS/PT (aPS/PT) or IgG fraction from healthy controls (control) (500 μg/ml) in the presence or absence of prothrombin (PT). (B) PBMCs treated with 231D or mouse control IgG (IgG) (10 μg/ml).

Murine monoclonal anti-prothrombin antibodies (51A6, 231D) and control murine IgG (IgG) were added to RAW264.7 cells at a concentration of 7.5 μg/ml with or without prothrombin (PT) (10 μg/ml), and then incubated for 4 h. After incubation, FITC-conjugated anti-mouse IgG antibody was added to the cell suspension and then analysed with a FACS analyzer. The vertical axes represent the number of cells and the horizontal axes represent the FITC fluorescence intensity.
RAW264.7 cells were exposed to the substances described below for 15 min. Prothrombin (PT), 231D and mouse control IgG (IgG) were added at a concentration of 10 μg/ml and LPS was added at a concentration of 100 ng/ml. The relative OD ratio of each sample was measured and calculated as described in the Materials and methods section. *P < 0.005, NS: not statistically significant.

PBMCs and the mouse monocyte cell line, RAW264.7, were exposed to stimulators for 5 h. Prothrombin (PT) and 231D were added at a concentration of 10 μg/ml and LPS was added at 100 ng/ml. Cells were pretreated with the p38-specific inhibitor SB203580 (1 μM) or its inactive analogue SB202474 (1 μM) for 30 min followed by treatment with stimulators. Vertical axes represent the relative expression levels of TF mRNA detected by real-time PCR. The bars represent the mean ± S.E. of three independent experiments. **P < 0.001, NS: not statistically significant. (A) The relative TF mRNA expression levels in PBMCs. (B) The relative TF mRNA expression levels in RAW264.7 cells.
PBMCs were pretreated for 72 h with siRNA of p38 MAPK or the control and subsequently exposed to stimulators for 5 h. Prothrombin (PT) and 231D were added at a concentration of 10 μg/ml and LPS was added at 100 ng/ml. The vertical axis represents the relative expression level of TF mRNA detected by real-time PCR. The bars represent the mean ± S.E. of three independent experiments. **P < 0.001, NS: not statistically significant.

231D and 51A6 significantly bound to the membranes of monocytes in the presence of prothrombin. The binding of 231D to the cell surface was clearly stronger than that of 51A6. This observation was similar to our previous report that 231D had stronger binding to the PS/PT complex than 51A6 [13], suggesting that the monoclonal antibodies bind to prothrombin complexed with phosphatidylerine on the cell surface.

Our data suggest that TF production induced by aPS/PT in procoagulant cells is mainly induced via activation of the p38 MAPK pathway, which is similar to past reports showing that p38 MAPK was the main pathway of aCL/β2GPI-induced cell activation. It is interesting that antibodies recognizing different proteins seem at least partially to utilize a common signalling pathway. Our findings are in agreement with the clinical observation that the manifestations of APS do not differ in patients with different antibody profiles.

Protein kinases are key regulators of cellular signalling, inflammation, cell differentiation and cell death. Thus they have been attractive targets for the treatment of neoplasms and inflammatory diseases [25–27].

p38 MAPK belongs to the MAPK signal protein family and is strongly activated by environmental stress or inflammatory cytokines such as TNF-α, IL-1β and IL-18 [28–30]. Consequently p38 MAPK activation is considered critical for physiological immune responses, and p38 MAPK dysfunction is related to the pathology of autoimmune diseases other than APS [31–33].

In the present study, phosphorylation of signal proteins, such as those in the MAPK protein family and serine/threonine kinases, was screened in aPS/PT-treated cells using a proteome array and major signals were quantitatively measured by ELISA tests. No proteins other than p38 MAPK were found to be phosphorylated. Further, specific p38 MAPK inhibitors or knockdown of p38 MAPK mRNA effectively inhibited procoagulant cell activation. Therefore p38 MAPK is suggested as a major signal protein for the activation of aPS/PT-induced procoagulant cells. Although a previous study showed that ERK activation was observed in cells treated with IgG fractions from APS patients [22], an ERK inhibitor did not abolish TF expression in procoagulant cells, suggesting that ERK does not play a major role in cell activation. We also did not detect the ERK phosphorylation in our aPS/PT-treated cells.

The two major aPLs, aCL/β2GPI and aPS/PT, are suggested to activate procoagulant cells primarily through p38 MAPK phosphorylation, therefore inhibition of p38 MAPK appears to be a promising modality for the treatment of APS. Since p38 MAPK contributes to various cell activities, its non-specific inhibition might result in severe complications. In fact, clinical trials of p38 inhibitors for a variety of diseases have been carried out; however, most of the trials encountered several complications and were unsuccessful [34]. A more realistic and practical strategy would be to target a more specific molecule involved in the activation of aPL-induced procoagulant cells.

Some reports have demonstrated possible receptors for aCL/β2GPI-induced cell activation on procoagulant cells. Annexin A2 is a receptor for tissue plasminogen activator and plasminogen that is found on the surface of ECs and monocytes, and on the brush-border membrane of placental syncytiotrophoblasts, all of which are recognized targets of pathogenic aPLs [35, 36]. Annexin A2 interacts with the β2GPI-aCL/β2GPI complex on EC and monocyte surfaces, mediating cell activation [37–39]. The involvement of annexin A2 in aPL-mediated pathogenic effects has been reported in vitro and in vivo [40, 41]. However, it is still not clear whether such a receptor is actually involved in cell activation because annexin A2 is not a transmembrane protein. Further, it has been proposed that activation of the signalling responses required another transmembrane adaptor protein(s) that associates with annexin A2 on the EC surface [42].

The Toll-like receptor (TLR) family, in particular, TLR-2 and TLR-4 [43–45], may also play a role in the interaction of the β2GPI-aCL/β2GPI complex [42]. Adhered β2GPI interacts with TLR-4 and aCL/β2GPI cross-links β2GPI and the TLR-4 complex, eventually triggering the signalling cascade activation. Moreover, TLR-4 is the putative adaptor protein for annexin A2 [38].

Further investigations have shown that megalin/gp330 [46] and apolipoprotein E receptor 2 [47–49] are putative receptors for aCL/β2GPI. Recently we identified the gelsoin/integrin α5β1 complex as a novel receptor of aCL/β2GPI [50].

In contrast to the intensive investigation of aCL/β2GPI thrombogenicity, no data are available on the mechanism of aPS/PT-dependent procoagulant cell activation. It is not yet known if aCL/β2GPI and aPS/PT have a common
cell surface receptor and upstream signals of p38 MAPK. However, we believe that our data are the first to show the critical pathway of the procoagulant state related to antibodies against prothrombin.

There are several reports showing the correlation between aPS/PT and APS-related pregnancy morbidity [51–53] that are subject to further investigation to clarify the molecular mechanism of the manifestation. Identification and comparison of the receptors for aPS/PT and aCL/β2GPI will help elucidate the pathogenicity of aPLs and the mechanisms of APS pathology.

**Rheumatology key messages**

- Phosphatidylserine-dependent aPT induced TF expressions on procoagulant cells in vitro.
- Similar to aCL, phosphatidylserine-dependent aPT induced cell activation via the p38 MAPK pathway.
- Cell activation via the p38 MAPK pathway may partially explain the pathogenesis of APS thrombosis.

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