Impaired fibrinolysis in patients with antiphospholipid syndrome

Fibrinolytic reactions involve the formation of plasmin generated from plasminogen and the hydrolytic cleavage of fibrin to fibrin degradation products by plasmin. Plasmin is one of the most potent enzymes and has a variety of biological activities; thus, the regulation of plasmin generation and activity is highly important to maintain the homeostatic balance in vivo. The intrinsic fibrinolysis pathway is mediated by plasminogen proactivator of blood, prourokinase, and contact activation components. Factor XIIa triggers this intrinsic fibrinolysis, as well as the intrinsic coagulation pathway. Congenital factor XII deficiency is correlated with thrombotic disease but not with bleeding tendency, suggesting the importance of intrinsic fibrinolysis. Plasmin formation from plasminogen by tissue plasminogen activator (tPA) is a key event in extrinsic fibrinolysis for thrombolysis of intravascular blood clots. Endothelial cells, when activated, secrete plasminogen activator inhibitor-1 (PAI-1), as well as releasing tPA, to suppress fibrinolysis by blocking tPA activity.

Impaired fibrinolysis is a contributing factor for the development of thrombosis, and the effect of antiphospholipid antibody (aPL) in the fibrinolytic system has been investigated, with controversial results probably due to the small size of the cohorts and heterogeneity of patient studies. Jurado et al. [1] suggested a hypofibrinolytic condition in patients with connective tissue diseases including APS, mainly related to increased PAI-1 levels. In this study, PAI-1 release by endothelial cell stimulation after venous occlusion was greatly enhanced in patients compared with healthy controls, but no difference was found in tPA release. To avoid the influence of the acute phase nature of PAI-1, Ames et al. [2] studied this issue in aPL-positive patients without underlying rheumatic diseases. They confirmed the up-regulation of PAI-1 levels in female patients with APS and further showed reduced tPA release by endothelial stimulation in those subjects, suggesting that tPA/PAI-1 balance was important in the development of thrombosis in some APS patients.

Several other reports pointed toward a hypofibrinolytic state in APS characterized by elevated PAI-1, suggesting a perturbation of endothelial cells with consequent fibrinolytic impairment [3–5], but no direct evidence was reported in the induction of PAI-1 by aPL.

The effect of lipoprotein (a) [Lp(a)] on the fibrinolytic system has been investigated. Lp(a) is an apoprotein which shares some sequence homology with plasminogen. Lp(a) is known to inhibit fibrinolytic activity not only by acting as an uncompetitive inhibitor of tPA [6] but also by increasing PAI-1 expression in endothelial cells [7]. This behavior suggests that Lp(a) has prothrombotic potential. Elevated plasma Lp(a) levels in patients with APS was reported by some authors [8,9]. We [8] analysed the relationship between Lp(a) levels and fibrinolytic state, showing that lower plasma D-dimer but higher PAI-1 levels in patients. These data suggested the deranged fibrinolysis was related to Lp(a) in patients with APS. Plasma Lp(a) is genetically determined, and it is possible that high Lp(a) levels in patients with APS might account for the impairment of fibrinolysis independently of the presence of aPL.

Structure and physiological function of β_2-glycoprotein-I

β_2-glycoprotein-I (β_2GPI), also known as apolipoprotein H, is a 50-kDa phospholipid-binding protein present in plasma at an approximate concentration of 200 μg/ml. Phospholipid-bound β_2GPI is one of the major target antigens for antiphospholipid antibodies present in patients with antiphospholipid syndrome (APS) [10–12]. β_2GPI has five homologous short consensus repeats and four glycation sites, forming an elongated fishhook-like three-dimensional structure. Domains of β_2GPI structurally resemble each other, except that domain V has an extra C-terminal loop and a positively charged lysine cluster. According to the crystal structure of human β_2GPI, it was proposed that this positively charged patch interacts with negatively charged phospholipid, with a flexible and partially hydrophobic loop inserted into the lipid layer when it binds to the cell surface [13,14]. β_2GPI also binds to other negatively charged molecules such as heparin, DNA, activated platelets, oxidized LDL, and apoptotic bodies.

Because negatively charged molecules trigger the intrinsic coagulation pathway, β_2GPI was proposed to be a natural anticoagulant. Indeed, β_2GPI has been reported to inhibit prothrombinase and tenase activity on platelets or phospholipid vesicles [15], to inhibit factor XII activation [16], and to modulate ADP-dependent activation of platelets [17]. Recently, β_2GPI has been shown to bind directly to factor XI and attenuate its activation [18]. On the other hand, β_2GPI exerts procoagulant activities by reduction of activated protein C [19]. Data from knockout mice suggests that β_2GPI may contribute in thrombin generation

in vivo [20]. In spite of these studies concerning roles of β_2GPI in coagulation/fibrinolysis system, genetic deficiency of β_2GPI is not a major risk factor of either thrombosis or bleeding in humans [21,22]. We identified two families of β_2GPI deficiency in a Japanese population, which is due to single nucleotide deletion and resulting frame shift in β_2GPI gene (β_2GPI Sapporo) [23]. All affected persons with this mutation were apparently healthy and had normal hemostatic markers [24]. In one family, all individuals with β_2GPI deficiency had increased serum LDL-cholesterol or smaller particle sizes of LDL, while the other had no apparent abnormality.

Physiological role of β_2GPI have been reported also in lipid metabolism, atherosclerosis, vascular biology, and apoptosis. Thus, β_2GPI has been recognized as a regulator in several physiological reactions.

Role of anti-β_2-glycoprotein-I antibodies

In patients with APS, pathogenic "antiphospholipid antibodies (aPLs)" are not directed against phospholipids itself, but against phospholipid-binding proteins, such as β_2GPI, prothrombin, annexin V, protein C or protein S. Among these, autoantibodies against β_2GPI have been detected frequently in patients with APS and therefore, have been most extensively investigated. Several studies using animal models have shown that anti-β_2GPI antibodies are not only a marker in APS but also play important roles in inducing thrombosis [25]. The mechanism of how anti-β_2GPI antibodies induce thrombosis remains unknown, although several hypotheses have been proposed. Coagulation/fibrinolysis imbalance in patients with APS or that induced by aPLs has been investigated. Inhibition of anticoagulant activity in β_2GPI has been suggested as one of the mechanisms. It was reported that anti-β_2GPI antibodies block the inhibitory effect of β_2GPI on factor Xa generation on activated platelets [26]. However, because genetic β_2GPI deficiency is not a risk for thrombosis, anticoagulant activity found in β_2GPI may not be essential in vivo.

Binding of anti-β_2GPI antibodies remarkably increases the affinity of β_2GPI for negatively charged phospholipid [27]. Increment of the affinity may modify the physiological function of β_2GPI, or inhibit the binding of other phospholipids-binding proteins. Activated protein C acts as an anticoagulant by degrading factors Va and VIIIa. Anti-β_2GPI antibodies are reported to inhibit both protein C activation and activated protein C [28]. This inhibition does not occur in the absence of β_2GPI [29].

We investigated the effects of anti-β_2GPI antibodies on extrinsic fibrinolysis using plasmin-sensitive synthetic substrate in the presence of tissue plasminogen activator (tPA), plasminogen, and soluble fibrin monomer [29]. Although β_2GPI does not affect the fibrinolytic activity, after suppression of fibrinolytic activity by PAI-1, physiological concentrations of β_2GPI recovered this suppression to some extent. Fibrinolytic activity was significantly decreased by addition of monoclonal anticardiolipin antibodies (ACA; EY1C8 and EY2C9) to this system in the presence of β_2GPI.

We developed a chromogenic assay system to measure activity of intrinsic fibrinolysis in euglobulin fractions, using kaolin as an activator [30]. In this system, addition of β_2GPI resulted in slight suppression of intrinsic fibrinolytic activity, which was enhanced by monoclonal anti-β_2GPI antibody. Most of all, euglobulin fractions prepared from patients with APS showed significantly lower activity of intrinsic fibrinolysis than those from healthy controls (Fig. 1). These data suggest that impairment of extrinsic and intrinsic fibrinolysis induced by pathogenic anti-β_2GPI antibodies is one of the

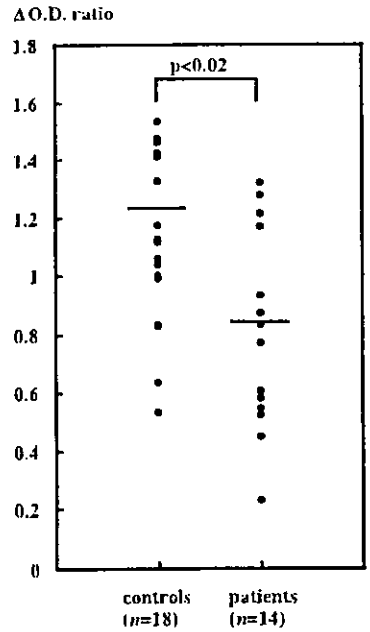

Figure 1 Intrinsic fibrinolytic activity in APS patients. The intrinsic fibrinolytic activities of euglobulin fractions from APS patients and healthy control subjects in the presence of 160 μg/ml of β_2GPI. These euglobulin fractions prepared from patients showed a significantly lower intrinsic fibrinolytic activity than those seen from control subjects.

mechanisms for the increased incidence of thrombosis in patients with APS.

Acceleration of atherosclerosis and activation of endothelial cells induced by anti-β_2GPI antibodies has been reported and growing evidence has accumulated in these fields. Activated endothelial cells overproduce tPA and PAI-1, resulting in the activation/regulation of the fibrinolytic system. The importance of atherosclerosis and cell-mediated events in APS will be discussed in other chapters. However, little data regarding aPL interaction with endothelium and fibrinolysis are available.

Negative feedback pathway in extrinsic fibrinolysis: cleavage of β_2GPI

β_2GPI is proteolytically cleaved between Lys-317 and Thr-318 in domain V (nicked β_2GPI), being unable to bind to phospholipids [31]. This cleavage is generated by factor Xa or by plasmin, the latter being more effective [32]. Once this cleavage occurs, β_2GPI loses the antigenicity for pathogenic aPLs [33]. Nicked β_2GPI has been identified in plasma of patients under conditions characterized by massive thrombin generation and fibrinolytic turnover, such as disseminated intravascular coagulation (DIC) [34], leukemia, or positive lupus anticoagulant. We have shown that the ratio of nicked β_2GPI against total β_2GPI is elevated in patients with cerebral infarction including asymptomatic lacunar infarct which is a condition with a lower level of thrombin generation and fibrin turnover. Nicked β_2GPI is a sensitive marker for the activation of fibrinolysis in vivo [35].

Plasminogen, the zymogen of plasmin, is a key molecule in the fibrinolytic reaction. Plasminogen consists of seven domains: one N-terminal peptide, five kringle domains bearing a lysine-binding site (LBS) with the capacity to bind fibrin as well as antifibrinolytic proteins carrying lysine, and one serine protease domain. Plasmin conversion from plasminogen by tissue plasminogen activator (tPA) occurs upon activation of extrinsic fibrinolysis for the thrombolysis against intravascular blood clots. Recently, we demonstrated that nicked β_2GPI, but not intact β_2GPI, binds to plasminogen with K_D of 0.37×10^{-6} M. According to a series of inhibition assay, this binding is presumably mediated by the interaction between the lysine cluster on the fifth domain of nicked β_2GPI and lysine-binding site on the fifth kringle domain of plasminogen. Nicked β_2GPI also suppressed plasmin generation up to 70% in the presence of tissue plasminogen activator,

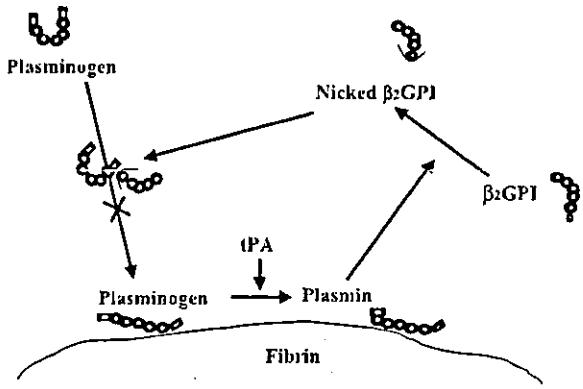

Figure 2 Upon formation of intravessel blood clot, plasminogen binds fibrin via kringle V and is activated by tissue plasminogen activator (tPA) into active plasmin. Then, the fibrinolytic system becomes active. Generated plasmin cleaves β_2GPI into nicked β_2GPI, that binds to plasminogen and inhibits its binding to fibrin, thus playing a role in a negative feedback pathway of extrinsic fibrinolysis.

plasminogen, and soluble fibrin monomer. Nicked β_2GPI interfered with the binding of plasminogen to fibrin, which is the first step in fibrinolysis. Thus, plasmin-generated nicked β_2GPI controls extrinsic fibrinolysis via a negative feedback pathway loop (Fig. 2). Although known physiological inhibitors of extrinsic fibrinolysis include α_2-antiplasmin and PAI-1, feedback inhibition by nicked β_2GPI is a novel mechanism in that it is generated only when the fibrinolytic system is activated.

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Antiprothrombin antibodies—are they worth assaying?☆

Tatsuya Atsumi*, Olga Amengual, Shinsuke Yasuda, Takao Koike

Department of Medicine II, Hokkaido University Graduate School of Medicine, N15 W7, Kita-ku, Sapporo 060-8638, Japan

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Abstract According to the preliminary classification criteria of the antiphospholipid syndrome (APS) (Sapporo Criteria), β2-glycoprotein I (β2GPI)-dependent anticardiolipin antibodies (aCL) and lupus anticoagulant (LA) are the only laboratory tests considered as criteria for the classification of the APS. Recently, antibodies against phosphatidylserine–prothrombin complex (aPS/PT) have been detected and these antibodies, rather than antibodies against prothrombin alone, are closely associated with APS and LA. We assessed the sensitivity and specificity of aPS/PT for the diagnosis of APS in our population of patients with a variety of autoimmune disorders and investigated whether aPS/PT could be used as diagnostic test in patients suspected of having APS. The study population comprised 219 patients with autoimmune diseases including 82 patients with APS and 137 without APS (55 systemic lupus erythematosus, 32 rheumatoid arthritis, 10 primary Sjogren's syndrome, 8 scleroderma, 5 Behcet's disease and 27 other rheumatic diseases). IgG/M aPS/PT were measured by ELISA using phosphatidylserine–prothrombin complex as antigen immobilized on ELISA plates in the presence of $CaCl_2$. IgG/M aCL were measured by standard methods and LA was detected by clotting assays. aPS/PT, aCL and LA were more frequently found in patients with APS (47, 46 and 69, respectively) than in those without APS (11, 19 and 29, respectively) (OR 95% [CI]; 15.4 [7.2–32.7], 7.9 [4.1–15.2, 19.8 [9.6–40.6], respectively]. The sensitivity of each assay for the diagnosis of APS was 57%, 56% and 86% with a specificity of 92%, 86% and 79%, respectively. aPS/PT and aCL have similar diagnostic value for APS, therefore, we propose that aPS/PT should be further explored, not only for research purposes, but also as a candidate of one of the laboratory criteria for the classification of the APS.

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* Corresponding author. Tel.: +81 11 706 5915; fax: +81 11 706 7710.
E-mail address: at3tat@med.hokudai.ac.jp (T. Atsumi).

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Markers for the diagnosis of antiphospholipid syndrome (APS)

Antiphospholipid syndrome is a clinical condition characterised by recurrent thrombotic events and pregnancy morbidity associated with the persistence of antiphospholipid antibodies (aPL). aPL are classified according to their in vitro method of detection; anticardiolipin antibodies (aCL) are measured by ELISA and lupus anticoagulant (LA) by clotting assays. However, the current concept of aPL includes antibodies directed against phospholipid-binding proteins involved in the coagulation system or their complexes with phospholipids.

Two decades have passed since the first description by Hughes et al. [1] of the association of aPL with thrombotic events, neurological disease, pulmonary hypertension, livedo, thrombocytopenia and recurrent miscarriages. The syndrome was named initially "anticardiolipin syndrome" and later "antiphospholipid syndrome" emphasising that aPL group rather than aCL alone was present in those patients.

In 1998, an International Consensus defined the preliminary classification criteria for definite APS [2]. The laboratory criteria stated that aPL (aCL and/or LA) must be positive on at least two occasions more than 6 weeks apart, to avoid false-positive aPL associated with acute infectious diseases.

LA are immunogloblins (IgG, IgM, IgA or their combination) that interfere with in vitro phospholipid-dependent tests of coagulation (prothrombin time [PT], activated partial thromboplastin time [APTT], kaolin clotting time [KCT], dilute Russell's viper venom time [dRVVT]). The term LA was first described in two patients with lupus. The term anticoagulant was used to describe the in vitro activity of the prolongation of the clotting test, not because of bleeding tendency.

LA determination in clinical practice

LA is detected by functional assays according to the recommendation of the Scientific and Standardisation Committee of the International Society of Thrombosis and Haemostasis (SSC of ISTH) [3]. The following approach has been proposed: (1) screening test to evaluate if phospholipid-dependent coagulation tests are prolonged, (2) mixing test to demonstrate that the prolongation of the clotting time is caused by an inhibitor present in plasma and (3) confirmation of the phospholipid nature of the inhibitory antibody by adding extra phospholipids or platelet neutralisation.

An accurate detection of LA is essential to facilitate the diagnosis of patients with thrombotic disorders. The identification of LA is, however, often difficult. No guidelines have been proposed on the nature and composition of the phospholipids used and several commercial tests for LA detection are available. Some publications have shown that the correlation among these tests is very low and, in fact, some laboratories rely on poorly responsive screening assays [4]. The LA confirming procedures are reasonable but practically difficult, as the definition of 'correction' by normal plasma or by phospholipids has not been established. Despite considerable efforts of SSC of ISTH for the last decade, the LA tests are still one of the most frustrating laboratory assays for routine clinical practice.

Prothrombin as a cofactor for LA

In 1991, Bevers et al. [5] highlighted the importance of aPT in causing LA activity. Some years later, Oosting et al. [6] showed that LA inhibited endothelial cell-mediated prothrombinase activity and IgG fraction containing LA activity bound to phospholipid–prothrombin complex. Therefore, prothrombin was identified as an antigen for autoantibodies with LA activity, as well as β2-glycoprotein I (β2GPI). Accordingly, it is widely accepted that antibodies to prothrombin and β2GPI are two major autoantibodies responsible for LA activity, antiprothrombin antibodies responsible for prothrombin-dependent LA and anti-β2GPI antibodies for β2GPI-dependent LA. Recently, it has been shown that specific neutralization enables differentiation between LA activity caused by anti-β2GPI antibodies and that by antiprothrombin antibodies. The addition of cardiolipin neutralised β2GPI-dependent LA but not prothrombin-dependent LA in an APTT based assay [7].

Prothrombin structure and in vitro function of antiprothrombin antibodies

Prothrombin (factor II) is a vitamin K-dependent glycoprotein present at a concentration of approximately 100 μg/ml in normal plasma. Mature human prothrombin consists of a single chain glycoprotein with a molecular weight of 72 kDa [8]. During its biosynthesis in the liver, prothrombin undergoes γ-

carboxylation. These γ-carboxyglutamic residues, known as the Gla domain, are located on fragment 1 of the prothrombin molecule. The Gla domain is essential for the calcium-dependent binding of phosphatidylserine to prothombin. A kringle domain containing two kringle structures and a carboxyl-terminal serine protease follows the Gla domain.

Prothrombin is physiologically activated by the prothrombinase complex (activated factor X, factor V, calcium and phospholipids). Once negatively charged phospholipids bind prothrombin, the prothrombinase complex converts prothrombin to thrombin, cleaving fibrinogen into fibrin [9]. In addition, thrombin binds thrombomodulin on the surface of endothelial cells and activates protein C, then exerts its anticoagulant activity by inactivating factor Va and depriving the prothrombinase complex of its cofactor. Because of this negative feedback pathway, prothrombin/thrombin behaves as "an indirect" anticoagulant.

Detection methods and clinical associations of antiprothrombin antibodies

Double diffusion, counterimmunoelectrophoresis and assays based on the impairment of prothrombin activation by antiprothrombin antibodies were the first techniques used for screening antiprothrombin antibodies, but they were not suitable for routine clinical practice.

In 1995, Arvieux et al. [10] described an ELISA for detection of antiprothrombin antibodies using prothrombin as antigen coated onto irradiated plates (anti-prothrombin alone; aPT-A). Since then, a number of clinical studies have investigated the clinical implications [11]. Some of those studies showed positive correlation between aPT-A and some of the clinical features of the APS including venous or arterial thrombosis [12–15], pregnancy loss [16,17] or clinical features of APS in general [18,19]. Other studies, however, failed to find a correlation between the presence of aPT-A and thrombosis [20–27].

Controversial results regarding aPT-A have also been reported in patients without autoimmune disease. In middle-aged men, high levels of aPT-A conferred a high risk of myocardial infarction or cardiac death and thrombotic events [28,29] but no correlations were found between aPT-A and thrombotic events in a large population of unselected patients with a history of venous thrombosis [30].

In 1996, antibodies directed to phosphatidylserine–prothrombin complex (or phosphatidylserine-dependent antiprothrombin antibodies; aPS/PT) were described in LA positive patients [31], and Galli et al. [24] reported that the assay using

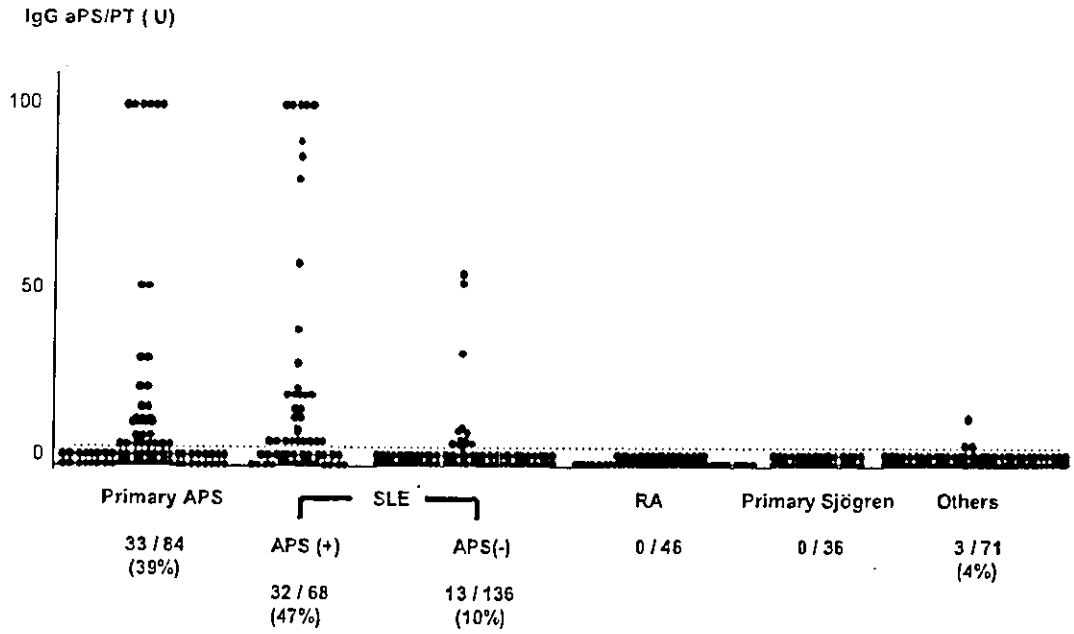

Figure 1 Prevalence of IgG phosphatidylserine-dependent antiprothrombin antibodies (aPS/PT) in 441 patients with autoimmune diseases. IgG aPS/PT were detected by enzyme-linked immunosorbent assay. The dashed line represents the cut-off for positivity. U: units, APS: antiphospholipid syndrome, SLE: systemic lupus erythematosus, RA: rheumatoid arthritis.

phosphatidylserine-bound prothrombin as antigen was more sensitive in demonstrating the presence of antiprothrombin antibodies than the system using prothrombin alone as an antigen.

The clinical studies described above were performed using the aPT-A ELISA. Our group [32] used the aPS/PT assay in a large population of patients with autoimmune diseases and found that IgG aPS/PT were highly prevalent in patients with APS compared with patients with other diseases (Fig. 1). We also showed that the detection of aPS/PT strongly correlated with the clinical manifestations of APS and with the presence of LA detected by dRVVT. Two major subpopulations of specific antibodies reactive with β2GPI or prothrombin are responsible for the LA activity in phospholipid-dependent coagulation tests. We showed that 95.6% of patients with aPS/PT had LA, thus, this new assay is a useful and easy tool to confirm the presence of LA. Therefore, the aPS/PT ELISA detects a subpopulation of antiprothrombin antibodies of significant clinical relevance.

Diagnostic value of aPS/PT for APS

In the present study, we assessed the sensitivity and specificity of aPS/PT for the diagnosis of APS and evaluate whether aPS/PT could be used as diagnostic test in patients suspected as APS. The investigated population comprised 219 patients with a variety of autoimmune diseases including 82 patients with APS and 137 without APS (55 systemic lupus erythematosus, 32 rheumatoid arthritis, 10 primary Sjogren's syndrome, 8 scleroderma, 5 Behcet's disease and 27 other rheumatic diseases). IgG/M aPS/PT were measured by ELISA using phosphatidylserine–prothrombin complex as antigen immobilised on ELISA plates in the presence of $CaCl_2$ as described [32]. Briefly, non-irradiated microtiter plates (Sumilon type S, Sumitomo Bakelite, Tokyo, Japan) were coated with 30 μl of 50 μg/ml of phosphatidylserine and dried overnight at 4 °C. To avoid nonspecific binding of proteins, wells were blocked with Tris-buffered saline containing 1% fatty acid-free bovine serum albumin (BSA) (A-6003; Sigma, St. Louis, MO) and 5 mM of $CaCl_2$ (BSA-Ca). After three washes, 50 μl of 10 μg/ml of human prothrombin (Diagnostica Stago, Asnieres, France) in BSA-Ca was added to the wells. After 1 h of incubation at 37 °C, plates were washed and 50 μl of patient serum diluted 1:100 in BSA-Ca was added in duplicated. Plates were incubated for 1 h at room temperature followed by the addition of alkaline phosphatase-conjugated goat anti human IgG or IgM and substrate. IgG/M aCL were measured by standard methods and LA was detected by clotting assays. Three clotting tests were performed for LA determination according to the guidelines recommended by the SSC of ISTH [3]. For the APTT test, a sensitive reagent with low phospholipid concentration was used for screening (Diagnostica Stago). A mixing text (plasma sample/normal pooled plasma 1:1, 1:4 and 1:9) and phospholipid addition test were used to confirm the presence of LA. A KCT test and confirmation via a mixing study were performed in the same manner. The dRVVT test was used to screen and confirm the presence of LA, using RVV-screen and RVV-confirm reagents (America Diagnostica, Greenwich CT).

APS/PT, aCL and LA were more frequently found in patients with APS (47, 46 and 69, respectively)

Table 1 Sensitivity and specificity of aPL tests for the diagnosis of APS

Assay	Sensitivity (%)	Specificity (%)
LA	86	79
aCL/β2GPI	56	86
aPS/PT	57	92

LA: Lupus anticoagulant, aCL/β2GPI: β2-glycoprotein I-dependent anticardiolipin antibodies, aPS/PT: phosphatidylserine dependent antiprothrombin antibodies.

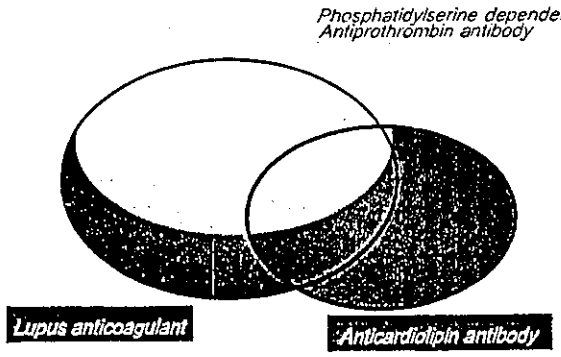

Figure 2 Distribution of antiphospholipid antibodies in patients with antiphospholipid syndrome (APS).

than in those without APS (11, 19 and 29, respectively) (OR 95% [CI]; 15.4 [7.2–32.7], 7.9 [4.1–15.2], 19.8 [9.6–40.6], respectively). The sensitivity and specificity of each assay for the diagnosis of APS are shown in Table 1. Both sensitivity and specificity of aPS/PT for the diagnosis of APS were comparable to those of aCL. In this series, 50 out of 58 patients with aPS/PT had LA (94.8%) (44/47 in APS group and 6/11 in the non-APS group). Most of the patients with aPS/PT had positive LA, thus, those data suggests that aPS/PT can also be one of the 'confirming' assays for LA. The distribution of aPS/PT, aCL and LA in patients with APS was shown in Fig. 2.

Apart from 'LA confirming' property, aPS/PT showed a very high specificity for the diagnosis of APS. Considering that aPS/PT and aCL have similar diagnostic value for APS, we propose that aPS/PT should be further explored, not only for research purpose, but also as a candidate of one of the laboratory criteria for the classification of the APS.

Conclusion

In conclusion, aPS/PT, as well as aCL/β2GPI, are useful tools for the diagnosis of APS. These assays should be performed in conjunction with the LA test. Additional and prospective studies on aPS/PT, however, are needed to establish the clinical relevance of these antibodies.

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CASE REPORT

Seiji Hashimoto · Yayoi Ogawa · Takashi Ishida
Toshio Mochizuki · Takao Koike · Hidetoshi Sato
Takahiro Ueda

Steroid-sensitive nephrotic syndrome associated with positive C1q immunofluorescence

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Abstract
A 21-year-old woman showed heavy proteinuria and edema. A light microscopic study of a renal biopsy specimen showed diffuse mild mesangial expansion, with borderline mesangial hypercellularity. An immunofluorescence study revealed dominant positive staining (3+) of C1q in the glomerular mesangium. Stainings for C3, C4, IgG, and IgM were weak or 1+. Staining for IgA was negative. Electron-dense deposits were present in the mesangial area. There was significant fusion of foot processes. There was no serological or clinical evidence of collagen disease. She was treated with oral prednisolone (initially, 40 mg/day). The proteinuria was alleviated and the patient remains in complete remission. The histopathological studies were compatible with C1q nephropathy, although the clinical outcome differed in a number of aspects. The clinical picture in the current patient appears to represent a very rare phenotype of nephritis.

Key words C1q nephropathy · Steroid · Nephrotic syndrome

S. Hashimoto (✉)[1] · T. Ishida · T. Ueda
Department of Nephrology Division, Sapporo General City Hospital, Sapporo, Japan

Y. Ogawa · H. Sato
Department of Pathology, Sapporo General City Hospital, Sapporo, Japan

T. Mochizuki · T. Koike
Second Department of Internal Medicine, Hokkaido University School of Medicine, Sapporo, Japan

Present address:
[1]NIH/NIDDK, Bldg. 10, Room 4D51, 10 Center Drive- MSC 1370, Bethesda, MD 20892, USA
Tel. +1-301-435-6582; Fax +1-301-435-6587
e-mail: seijinih@med.hokudai.ac.jp

Introduction

In 1985, Jennette and Hipp[1] described C1q nephropathy (C1qNP) as a distinct pathological entity. This entity is characterized by an immune complex glomeulonephritis and complement deposits, most notably C1q, and by the absence of clinical and laboratory evidence of systemic lupus erythematosus (SLE). On light microscopy studies, several morphological patterns have been described. C1qNP commonly presents with nephrotic-range protenuria and has a poor response to steroids in older children and young adults. Patients may have decreased renal function at presentation; however, progression to endstage renal disease is slow. In 1982, five patients with presentation similar to that later described by Jennette and Hipp[1] were reported by Jones and Magli,[2] who used the terms "mesangiopathic glomerulonephritis with 'full-house' immunofluorescence".

We report here that prednisolone treatment of mesangial C1q deposits in a nephrotic patient alleviated proteinuria and caused full remission in the patient. The disease was unrelated to SLE or other collagen diseases. We compare the features in our patient with those of previously reported cases of C1qNP.

Case report

A 21-year-old woman was admitted to Sapporo General City Hospital on June 26, 2000, because of severe edema. Prior to admission she had a 10-day history of episodes of sudden-onset edema. She had no pertinent family history and no previous illnesses. Blood pressure was 110/72 mmHg, pulse rate was 48 beats/min with regular sinus rhythm, and body temperature was 36.0°C. Investigation confirmed microscopic hematuria, with heavy urinary protein leak, of 11 g/24 h. Urinalysis showed 3+ for protein and 1+ for blood on dipstick examination. Microscopic evaluation demonstrated three to five red blood cells and hyaline casts per high-power field (HPF). Relevant laboratory

serum data were a hematocrit of 44%, and a hemoglobin concentration of 15.0 g/dl. The leukocyte count was 7500/μl. The platelet count was 291000/μl. Urea nitrogen was 12 mg/dl; creatinine, 0.5 mg/dl; total protein, 4.1 g/dl; albumin, 1.9 g/dl; C-reactive protein, 0.1 mg/dl; and total cholesterol, 472 mg/dl. Immunoglobulin (Ig) G was 718 mg/dl, IgA was 287 mg/dl, and IgM was 236 mg/dl. Complement C3 was 130 mg/dl (normal range, 60 to 116 mg/dl), C_4 was 24.4 mg/dl (normal range, 12 to 32 mg/dl), and assay for total complement (CH50) was 40.6 U/ml (normal range, 34 to 49 U/ml). C1q was 1.5 μg/ml (normal range, 0 to 3.0 μg/ml) and C3d was 6.0 μg/ml (normal range, 0 to 13.0 μg/ml). Interleukin-6 was 2.4 pg/ml (normal range, 0 to 4.0 pg/ml). Antinuclear antibody and anti-DNA antibody were both negative. She had no abnormality on immunological investigations, and various kinds of autoantibodies (anti-nuclear antibody, anti-DNA antibody, anti-ribonucleoprotein antibody, anti-Sm antibody, and anti-Scl70 antibody) were not detected. Rheumatoid factor and LE test were negative.

Eighteen glomeruli were identified in the sections submitted for light microscopic examination. The specimens showed neither global sclerosis nor focal segmental lesions. There was mild diffuse mesangial expansion, with borderline mesangial hypercellularity (Fig. 1). There were no tubulointerstitial or arteriolar changes. By immunofluorescence, dominant positive staining (3+) of C1q was found in the glomerular mesangium and capillary loops. Stainings for C3, C4, IgG, and IgM were weak or 1+. Staining for IgA was negative (Fig. 2). C1q antibody was obtained from ICN/Cappel (Durham, NC, USA). One glomerulus was identified in thick sections submitted for electron-microscopic survey. Electron-dense deposits were present in the mesangial area. The glomerulus showed minimal mesangial proliferation, with increased mesangial matrix. There was foot process fusion (Fig. 3).

Following the administration of prednisolone (initially, 40 mg/day), the proteinuria and hematuria were alleviated. After 3 weeks, all urinary findings were normal. Although

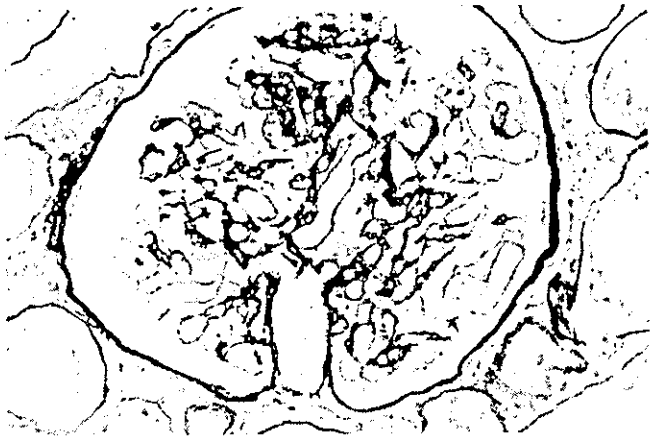

Fig. 1. Renal biopsy specimen, showing borderline mesangial hypercellularity. HE, ×40

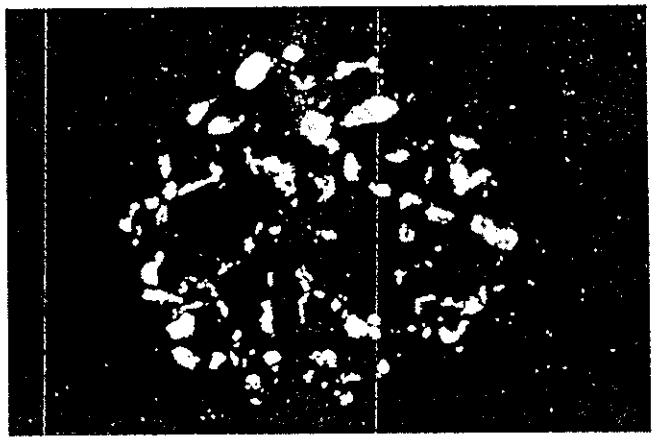

Fig. 2. Immunofluorescence photomicrograph of a glomerulus reacted with anti-C1q antibody, showing extensive, predominantly mesangial, C1q localization. ×400

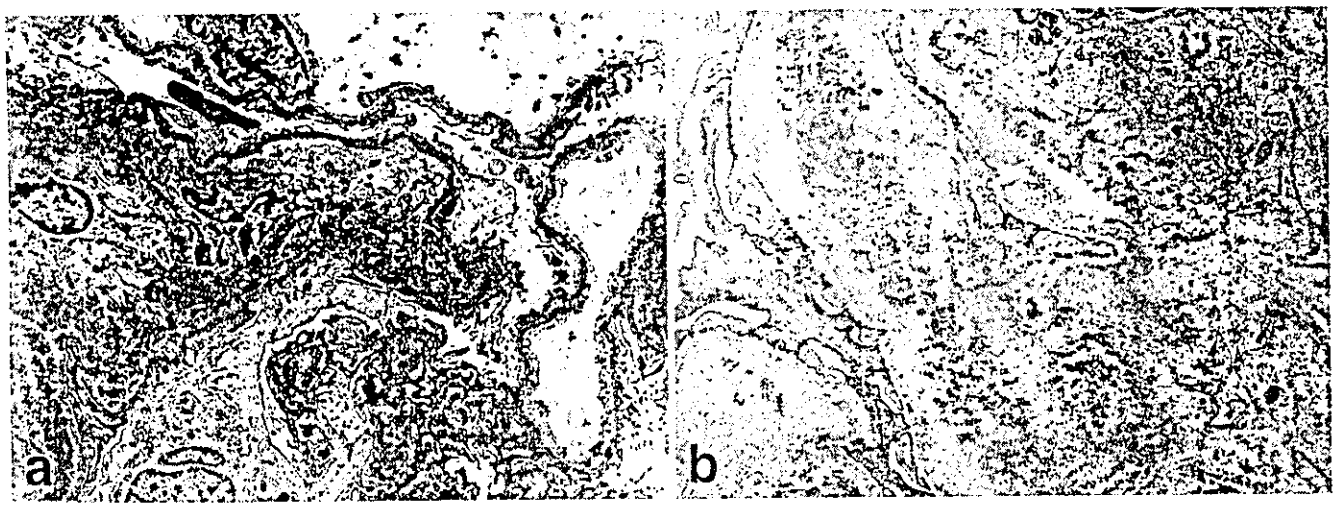

Fig. 3. a Electron micrograph of a glomerular capillary and adjacent mesangium. b Electron micrograph demonstrating the presence of extensive electron-dense deposits. a ×4500; b ×15000

the prednisolone dose was gradually reduced, the patient has remained in complete remission for more than 2 years. No clinical evidence of SLE or other collagen diseases was obtained over this time period.

Discussion

Jennette and Hipp[1] proposed that C1qNP was a distinct clinical entity causing steroid-resistant nephrotic syndrome in the absence of SLE. This initial report suggested that C1qNP patients were young and had good renal function at presentation; however, they did not show remarkable clinical features. Because C1qNP is a rare disease, its clinical outcome is still unclear.

In histopathological studies, C1qNP is characterized by extensive and dominant C1q mesangial deposition. Several morphological patterns have been described, ranging from no histological glomerular alterations (NHA) to focal and diffuse mesangial proliferative changes (MP).

The second report of C1qNP was presented by Iskandar et al.[3] They were the first to report C1qNP of focal segmental glomerulosclerosis (FSGS) type, and it seemed that the clinical outcome showed subtle differences from the initial report. They showed that patients with C1qNP may have decreased renal function at presentation, but that progression to endstage renal disease is slow. Some of these C1qNP patients with NHA responded to pulse steroid treatment, showing remission, whereas those patients with FSGS and MP remained a therapeutic problem.

In the third report of C1qNP, Davenport et al.[4] reported the clinical outcome of four patients with C1qNP; three of their four patients improved spontaneously. Histological evaluation of these patients showed membranoproliferative glomerular nephritis, membranous glomerular nephritis, and MP. The remaining patient was initially treated with high-dose steroid, but without success; in this patient, with FGS-type pathology, the urinary protein loss resolved following additional treatment with cyclosporine. There are now multiple reports of purported C1qNP that differs considerably from the previously reported cases.[5–7] These reports suggest that C1qNP has various light microscopic findings, and that these findings do not always determine the clinical features. However, C1qNP shows steroid resistance even if the condition is not comparable to that in the initial report. We report here that our patient's histological and immunofluorescence findings were compatible with C1qNP, and that the condition was resolved promptly with general steroid medical treatment.

Our patient's histopathological analysis showed similarities to the type of C1qNP with MP. Complete remission was achieved with the initial typical course of oral steroid therapy. Another case of steroid-sensitive C1qNP has been reported recently;[8] however, the patient's clinical course and conditions were quite different from the findings we reported here. Most notably, the patient reported by Ekim M et al. was a child with severe allergy, and a high serum Ig E level and eosinophilia were detected in this child. We are uncertain about any relationship between C1qNP and severe atopic dermatitis. Nevertheless, steroid treatment effectively alleviated both the C1qNP and the atopic dermatitis.

More recently, a C1qNP report was published by Markowitz et al.[9] They concluded that C1qNP was a variant of FSGS. However, the initial report of Jennette and Hipp[1] did not show that the pathological changes in C1qNP resembled those in FSGS. Segmental lesions, capillary collapse, increasing of epithelial cells, and hyalinization are the characteristics of FSGS. These characteristics were not indicated by all past C1qNP reports, and were not found in our patient, either. C1qNP shows a variety of features. However, the pathological changes in FSGS do not seem to be similar to those in C1qNP. The differences are also clear clinically. Rydel et al.[10] reported that 50% of FSGS patients achieved a remission by 3.7 ± 2 months (33% complete remission and 17% partial remissions), with all patients responding within 9 months. Generally, C1qNP is steroid-resistant as compared with FSGS, and it is well known that nephrotic FSGS patients have a poor prognosis. In contrast, C1qNP progresses slowly.

In conclusion, we report a case in which histological and immunofluorescence evidence in renal biopsy specimens was compatible with C1qNP. Although there are controversial results with response to therapy and prognosis, our patient had a favorable response to steroid therapy, with complete remission. Also, our case was not categorized as FSGS. We consider that the question concerning histopathological analysis and the clinical future in patients with C1qNP still leaves room for discussion. We expect that further cases will permit us to proceed with research in this category in the future.

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The p38 mitogen-activated protein kinase (MAPK) pathway mediates induction of the tissue factor gene in monocytes stimulated with human monoclonal anti-β_2Glycoprotein I antibodies

Miyuki Bohgaki, Tatsuya Atsumi, Yumi Yamashita, Shinsuke Yasuda, Yoshie Sakai, Akira Furusaki, Toshiyuki Bohgaki, Olga Amengual, Yoshiharu Amasaki and **Takao Koike**

Department of Medicine II, Hokkaido University Graduate School of Medicine, N-15 W-7, Kita-ku, Sapporo 060-8648, Japan

Keywords: anti-cardiolipin antibody, anti-phospholipid antibody, anti-phospholipid syndrome, thrombosis

Abstract

The anti-phospholipid syndrome (APS) is characterized by thrombosis and the presence of anti-phospholipid antibodies (aPL). Tissue factor (TF), the major initiator of the coagulation system, is induced on monocytes by aPL *in vitro*, explaining, in part, the pathophysiology in this syndrome. However, little is known regarding the nature of the aPL-induced signal transduction pathways leading to TF expression. In this study, we investigated aPL-inducible genes in PBMC using cDNA array system and real-time PCR. Our results indicated that the mitogen-activated protein kinase (MAPK) pathway was related to TF expression when PBMCs were treated, in the presence of β_2Glycoprotein I (β_2GPI), with human monoclonal anti-β_2GPI antibodies [β_2GPI-dependent anti-cardiolipin antibodies (aCL/β_2GPI)]. Western blotting studies using monocyte cell line (RAW264.7) demonstrated that p38 MAPK protein was phosphorylated with nuclear factor κB (NF-κB) activation by monoclonal aCL/β_2GPI treatment, and that SB203580, a specific p38 MAPK inhibitor, decreased the aCL/β_2GPI-induced TF mRNA expression. The p38 MAPK phosphorylation, NF-κB translocation and TF mRNA expression triggered by aCL/β_2GPI were abolished in the absence of β_2GPI. These results demonstrated that the p38 MAPK signaling pathway plays an important role in aPL-induced TF expression on monocytes and suggest that the p38 MAPK may be a possible therapeutic target to modify a pro-thrombotic state in patients with APS.

Introduction

Anti-phospholipid syndrome (APS) is a clinical condition characterized by recurrent thrombotic events and/or pregnancy morbidity associated with the persistence of anti-phospholipid antibodies (aPL) (1). Anti-cardiolipin antibodies (aCL) are members of the aPL family, a large and heterogeneous group of circulating Igs arising in a wide range of infectious and autoimmune diseases, particularly systemic lupus erythematosus (1). Since the early 1980s, the interest in aCL has exponentially increased due to their association with clinical manifestations of APS (2, 3).

aCL are detected by immunological assays (radioimmunoassay and ELISA) (2, 4, 5). Many studies have indicated that antibodies against β_2Glycoprotein I (β_2GPI) are one of the predominant antibodies detected with aCL assay in APS patients (6–12). APS-related aCL recognizes the epitope(s) on the β_2GPI molecule when β_2GPI interacts with a lipid membrane composed of negatively charged phospholipids [β_2GPI-dependent anti-cardiolipin antibodies or antibodies against cardiolipin/β_2GPI complex (aCL/β_2GPI)] (11). In the past decade, many studies have investigated the pathophysiology of thrombosis in APS and considerable interest has focused on the role of aPL as a clue to mechanisms related to thrombosis (13). Results of intensive research works have significantly advanced understanding of the mechanisms by which these antibodies may play a direct role in clot formation. Classically, *in vitro* evidence suggests that aCL/β_2GPI are involved in hemostatic abnormality. β_2GPI interacts with negatively charged phospholipids involved in the coagulation process, having both pro-coagulant and anticoagulant properties. β_2GPI suppresses the thrombomodulin–protein C system

Correspondence to: T. Atsumi; E-mail: at3tat@med.hokudai.ac.jp

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(14), factor X, XI and XII activation (15–18) and pro-thrombinase activity (19). Antibodies against β_2GPI may modify the properties of β_2GPI and favor a pro-thrombotic state.

However, individuals with β_2GPI deficiency do not have a thrombotic tendency, thus aCL/β_2GPI-associated thrombosis cannot be merely explained by 'β_2GPI insufficiency' (20, 21). Investigators turned their focus on functions of endothelial or other cells which might be modified by aPL. In this scenario, β_2GPI serves as a 'co-factor' to prepare receptors for auto-antibody binding to cells. In this case, irrespective of the functions of β_2GPI itself, auto-antibodies against β_2GPI may alter the bound-endothelium properties from 'anti-thrombotic' to 'pro-thrombotic', leading to the production of pro-coagulant substances such as tissue factor (TF) (22, 23), vascular cell adhesion molecule-1, intercellular adhesion molecule-1, E-selectin (24–26), plasminogen activator inhibitor-1 or endothelin-1 (27).

TF is the major initiator of the extrinsic coagulation system (28), functioning as the protein co-factor for the plasma serine protease, activated factor VII (FVIIa) (29, 30). Induced TF forms a complex with FVIIa that triggers the blood clotting cascade by activating factors IX and X, leading to thrombin generation (29). In normal conditions, TF is not expressed on intra-vascular cells (28) but it can be induced under stimuli such as LPS (31), tumor necrosis factor-alpha (TNF-α) (32, 33) and IL-1 (34). Evidence has supported the role of TF pathway in the pathogenesis of aPL-related thrombosis (22, 23, 35–37). Preliminary experimental data showed that sera or IgG fraction containing aCL induce TF-like pro-coagulant activity in endothelial cell (38, 39) or PBMC (40, 41). We and others demonstrated the up-regulation of TF pathway in patients with APS (35, 42, 43). Patients with APS have a pro-thrombotic state as evidenced by elevated basal thrombin generation (37, 44). Increased TF expression on endothelial cell or monocytes induced by aPL could, in part, be responsible for the hyper-coagulability and explain the existence of thrombosis in both the arterial and venous circulation that characterizes those patients.

On the other hand, only a few data have been published regarding the intracellular pathway in aPL-induced expression of TF or other pro-coagulant substances. We screened gene expression of molecules involved in signal transmission in aCL/β_2GPI-induced TF expression using cDNA array system and demonstrated the significance of the p38 mitogen-activated protein kinase (MAPK) phosphorylation procedure in such cell activation.

Methods

Isolation and preparation of cells

Venous blood was collected in heparin from healthy donors. PBMCs were isolated on Ficoll-Paque plus® gradient centrifugation (Amersham Biosciences Corp., Piscataway, NJ, USA). PBMCs were washed with RPMI-1640 medium (Sigma Chemical Co., St Louis, MO, USA) supplemented with 10% FCS (GIBCO BRL, Paisley, UK) containing penicillin and streptomycin (RPMI-10 medium) once at 20°C, 400 × *g*, for 5 min and twice at 4°C, 400 × *g*, for 5 min. PBMCs were then re-suspended in RPMI-10 medium and counted using the trypan blue dye exclusion method. Murine RAW264.7 (American Type Culture Collection registration no. #TIB-71) monocytes were maintained in a 5% CO_2 atmosphere at 37°C in DMEM (GIBCO BRL) supplemented with 10% FCS containing penicillin and streptomycin.

Proteins

Human β_2GPI was purified from normal sera, as described (11), and the purity was confirmed using SDSP. Fatty acid-free BSA was obtained from Sigma-Aldrich Inc. (A-6002; St Louis, MO, USA). Two human IgM monoclonal aCL/β_2GPI (EY2C9 and TM1G2) and one control monoclonal IgM lacking aCL/β_2GPI activity (TM1B9) were used in this study. EY2C9 and TM1G2 are IgM class human monoclonal aCL/β_2GPI established from APS patients with high titers of aCL/β_2GPI (45). The characteristic of EY2C9 and TM1G2 is that these are mAbs that bind to the cardiolipin–β_2GPI complex but not to cardiolipin alone (45). In the absence of cardiolipin, they do not recognize β_2GPI immobilized on the plain ELISA plate, but do bind to β_2GPI coated on an oxidized ELISA plate. The epitope mapping by phage-displayed peptide library demonstrated W^{235} in the fourth domain of β_2GPI as a key amino acid residue at the epitopic center (46). Therefore, we consider that these mAbs represent autoimmune aCL/β_2GPI found in patients with APS. The mAbs, when purified from serum-free medium culture supernatant, showed a single band on SDSP. LPS were intensively removed from these antibody preparations with DetoxiGel® (Pierce, Rockford, IL, USA) and were not detected using the *Limulus* amebocyte lysate assay (Limulus ES-II Single Test Wako: Wako, Osaka, Japan).

RNA extraction and cDNA array analysis

Total RNAs were isolated from PBMC or RAW264.7 using TRIzol® reagent (Invitrogen, Carlsbad, CA, USA), and stored at −80°C until use. Poly(A) RNA was isolated from total RNA (100 μg) using a MagExtractor® (TOYOBO, Osaka, Japan), and poly(A) RNA (2 μg) was reverse transcribed by ReverTraAce (TOYOBO) in the presence of cDNA synthesis primers and biotin–16-deoxyuridine triphosphate (TOYOBO), according to the manufacturer's instructions. cDNA array analysis was performed using human cDNA expression filters [Human Immunology Filters (TOYOBO), on which 621 species of human cDNA fragments and housekeeping genes were duplicately spotted]. Genes on the filter are shown on the web site http://www.toyobo.co.jp/seihin/xr/product/genenavi/genenavigator.html. Hybridization and subsequent cDNA array analyses were done as described (47), but with some modification. Briefly, cDNA array filters were pre-hybridized with PerfectHyb® solution (TOYOBO), and then hybridized with a biotin-labeled cDNA probe overnight at 68°C. After washing under high-stringency conditions, specific signals on the filters were visualized using Phototope-Star Detection Kits (New England Biolabs, Beverly, MA, USA), according to the manufacturer's recommendation. Fluorescence signals for mRNA expression levels were obtained using a Fluor-S Multiimager system (Nippon Bio-Rad Laboratories, Tokyo, Japan) and intensity of the signals was determined using ImaGene 4.2 software (BioDiscovery, Los Angeles, CA, USA).

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used as an internal control to normalize the mRNA abundance. The signal intensity among filters was compared in an E-Gene Navigator Analysis (GeneticLab, Sapporo, Japan) and expressed as mRNA expression index to the intensity of the internal GAPDH gene.

Quantitative TaqMan real-time PCR

Real-time PCR amplification and determination were done using the ABI PRISM 7000® Sequence Detection System (Applied Biosystems, Foster City, CA, USA) and gene-specific sets of TaqMan Universal PCR Master Mix® and Assays-on-Demand® Gene Expression probes (Applied Biosystems). A standard curve for serial dilutions of GAPDH was generated using a standard method provided by the manufacturer (Applied Biosystems), and was used to determine the amounts of cDNA transcripts.

Western blot analyses

For western blot analysis of p38 phosphorylation and nuclear nuclear factor κB (NF-κB) translocation, PBMC or RAW264.7 cells were treated with monoclonal aCL/β_2GPI (10 μg ml^{-1}) or control mAb in the presence/absence of β_2GPI (50 μg ml^{-1}) in serum-free medium, or 10% FCS medium at 37°C, followed by a preparation of cytosolic and nuclear proteins using a proteome extraction kit (Merck, Darmstadt, Germany). The cell lysates were resolved on 10% SDSP gel and then transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). The membranes were blocked with PBS containing 5% non-fat dry milk (Nestle USA, Inc., Solon, OH, USA) and 0.1% Tween–PBS buffer for 1 h, and probed with the rabbit polyclonal anti-phospho-MAPK antibody using a Phospho-MAPK family antibody sampler kit (Cell Signaling Technology, Inc., Beverly, MA, USA) overnight at 4°C. For some experiments, the blots were stripped and re-probed with polyclonal anti-MAPK antibody that recognizes both activated and non-activated MAPK proteins using an MAPK family antibody sampler kit (Cell Signaling Technology, Inc.). After three washes in 0.1% Tween–PBS buffer, the membranes were exposed to HRP-conjugated goat anti-rabbit antibodies at room temperature. Immunoreactive proteins were visualized using enhanced chemiluminescence assay (Amersham Biosciences Corp.). For analysis of NF-κB, nuclear lysates from the stimulated cells were blotted and reacted with an anti-NF-κB antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), the NF-κB antigen was visualized in the same fashion. In some experiments, the p38 MAPK inhibitor, SB203580 (Calbiochem, La Jolla, CA, USA), and the negative control for p38 MAPK inhibition studies, SB202474 (Calbiochem), were dissolved in dimethyl sulfoxide before addition to the culture medium.

Surface staining for FACS analysis

Surface aCL/β_2GPI binding on RAW264.7 was analyzed using FACSCalibur (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) with the CellQuest program. The cultured cells were washed with FACS buffer (2% BSA, 0.1% NaN_3 and PBS), and treated with 50 μg ml^{-1} of β_2GPI at room temperature for 10 min, followed by exposure to EY2C9 or TM1G2 (final concentration, 20 μg ml^{-1}) for 30 min on ice. After washing twice with FACS buffer, cells were stained with FITC-conjugated goat anti-human IgG/IgM antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) for 30 min on ice. After further two washes with FACS buffer, cells were subjected to FACS analysis. For each sample, data from 10 000 volume-gated viable cells were collected.

Results

Identification of monoclonal aCL/β_2GPI antibody-inducible genes

The effect of monoclonal aCL/β_2GPI on PBMC was screened utilizing cDNA arrays and mRNA expression of genes associated with the human immune system, including transcription factors, effector molecules and cytokines shown in Fig. 1. As a whole, the most increased mRNA expression by aCL/β_2GPI treatment in cell signaling was detected in 2-h incubated cells (Fig. 1A). In the 2-h cDNA array analysis in PBMC exposed to EY2C9 and control IgM, mRNA related to MAPK pathway such as p38 regulated/activated protein kinase, TNF receptor-associated factor 6 (TRAF6), Sp-1, SAPK4 (p38δ) and MAPK-activated protein kinase (MAP-KAPK)-3 increased >2-fold in EY2C9-treated cells compared with those treated with control IgM (Fig. 1A and Fig. 1B, left). In contrast, the expression of other signaling pathway molecules (Fig. 1B, right), such as tyrosine kinase, protein kinase C and Akt kinases, increased <2-fold. The expression of pro-inflammatory cytokine genes such as TNF-α and IL-1β, known to be regulated by the MAPK pathway, were enhanced up to 2- to 4-fold in the cDNA array (Fig. 1C). To confirm the results of cDNA array screening and previously reported TF mRNA expression by stimulation of aCL/β_2GPI, a real-time PCR method was applied. Both EY2C9 and TM1G2 stimulation increased TF (Fig. 2A), TNF-α (Fig. 2B) and IL-1β (Fig. 2C) mRNA expression. Up-regulated TF mRNA expression was also detected using real-time PCR in the monocyte cell line RAW264.7 after treatment with EY2C9 or TM1G2 (data not shown).

Identification of p38 MAPK phosphorylation as a pathway of aCL/β_2GPI activation

According to the results of cDNA array analysis, some MAPK-related molecules were up-regulated in PBMC by stimulation with aCL/β_2GPI, therefore, we further asked if the cDNA array results were associated with MAPK pathway activation. Non-phosphorylated forms of MAPKs (total MAPKs) were detected in unstimulated RAW264.7 cells. Cells treated with EY2C9 showed phosphorylation of p38 MAPK, which persisted for at least 60 min (Fig. 3). In contrast with ERK and JNK phosphorylation by LPS, neither ERK nor JNK pathway was activated with EY2C9 stimulation (Fig. 3).

Reduction of p38 MAPK activation in aCL/β_2GPI-mediated up-regulation of TF expression

To elucidate the role of p38 MAPK in TF mRNA expression, the effect of p38 MAPK inhibitors on the cells stimulated with monoclonal aCL/β_2GPI was examined. The p38-specific inhibitor, SB203580, entirely hampered p38 MAPK phosphorylation in RAW264.7 treated with EY2C9 (Fig. 4A), but SB202474, the inactive analogue of SB203580, did not affect

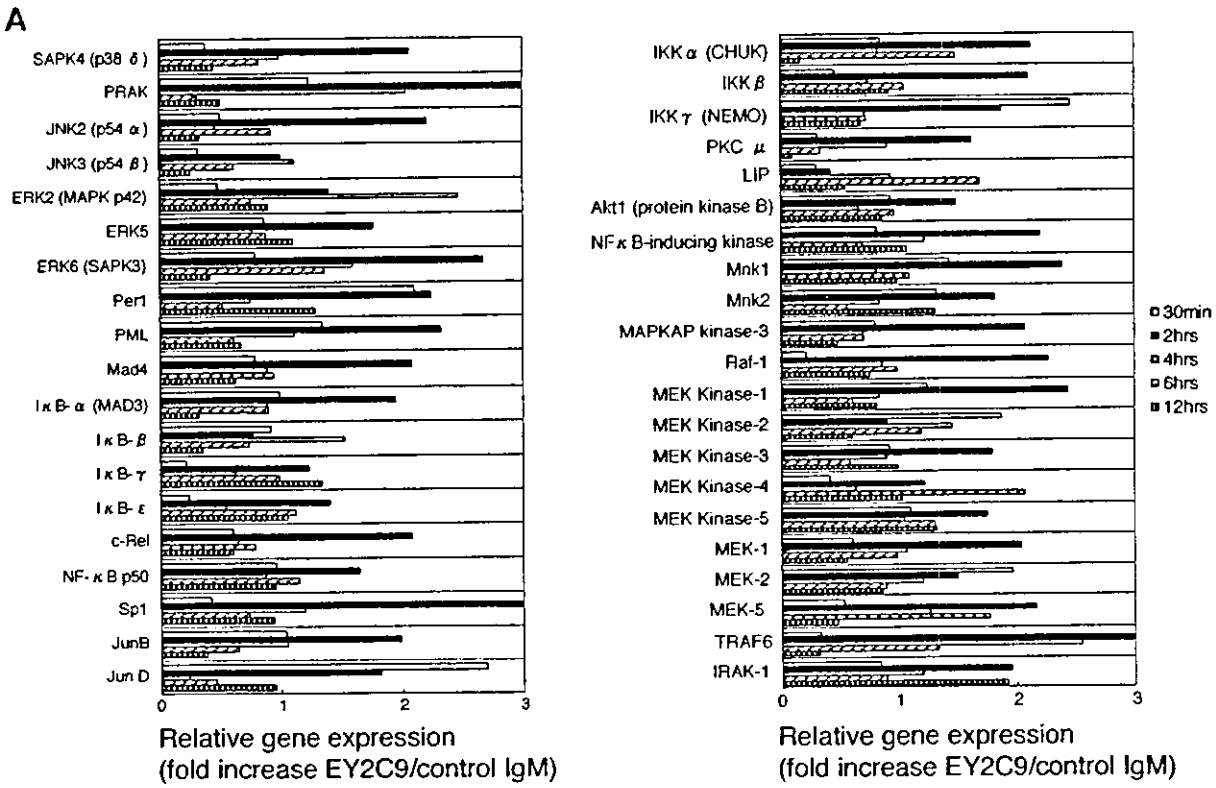

Fig. 1. Screening of up-regulated genes induced by monoclonal aCL/β_2GPI in PBMC: mRNA level of genes encoding various intracellular signaling transcription factors and molecules. PBMCs were stimulated with monoclonal IgM aCL/β_2GPI (EY2C9) or control antibody at 30 μg ml^{-1} for 30 min and 2, 4, 6 and 12 h. mRNA expression levels were analyzed by cDNA array as described in Methods. A. mRNA expression level of selected genes encoding mainly those associated with the MAPK pathway. These panels show fold increases in the mRNA expression level in EY2C9-stimulated cells compared with that in control IgM-stimulated cells. Increased mRNA expression was detected in 2-h incubated cells. B. Two-hour cDNA array analysis. mRNA expression level of selected genes encoding mainly those associated with the MAPK pathway (left panel) and various other signaling pathways (right panel). Genes are put in order of mRNA expression levels in each panel. PBMCs were treated with monoclonal IgM aCL/β_2GPI (EY2C9) or control antibody at 30 μg ml^{-1} for 2 h. mRNA expression levels were analyzed using cDNA array as described in Methods, and shows fold increase of the mRNA expression level in EY2C9-stimulated cells compared with that in control IgM-stimulated cells. MAPK-related molecule mRNA expressions are higher than other cell signal transduction molecules. C. mRNA expression level of some selected genes encoding cytokines and chemokines. This panel shows fold increases in the mRNA expression level in EY2C9-stimulated cells for 2 h compared with that in control IgM-stimulated cells for 2 h.

p38 phosphorylation. Addition of SB203580 to cells stimulated with EY2C9 or TM1G2 decreased TF mRNA expression up to 75% (Fig. 4B).

β_2GPI dependency of p38 MAPK phosphorylation and TF induction

To evaluate the β_2GPI dependency of EY2C9 stimulation, we established RAW264.7 cells adapted to serum-free medium and treated these cells with EY2C9 in the absence/presence of human β_2GPI. In the cells stimulated by EY2C9, p38 MAPK phosphorylation was observed in the presence of β_2GPI either in serum-free medium or in medium supplemented with 10% FCS, but there was no apparent effect of this monoclonal aCL/β_2GPI in the absence of β_2GPI (Fig. 5A). In addition, NF-κB was increased in the nuclear fraction after stimulation with EY2C9 in the presence of β_2GPI (Fig. 5A). Furthermore, EY2C9 or TM1G2 induced RAW264.7 TF mRNA expression in a β_2GPI-dependent manner, whereas β_2GPI had little effect on LPS-induced expression of TF mRNA (Fig. 5B). In FACS analysis, EY2C9 bound to the cells in the presence of β_2GPI, but no binding of EY2C9 was found in the absence of β_2GPI (data not shown).

Discussion

In the present study, we demonstrated that the p38 MAPK-dependent signaling pathway participates in aPL-mediated TF expression. The multi-screening using the cDNA array system combined with real-time PCR analysis indicated that the MAPK pathway was related to TF expression when cells were treated with monoclonal aCL/β_2GPI. We performed western blotting studies to confirm the result of cDNA array at protein level that p38 MAPK protein was phosphorylated. The specific p38 MAPK inhibitor decreased TF mRNA expression by aCL/β_2GPI stimulation, suggesting a crucial role of the p38 MAPK pathway in this system.

The association between aPL and the occurrence of thrombosis is widely recognized. The effect of aCL in the inhibition of natural anticoagulant systems, the impairment of fibrinolytic activity and the direct effect of these antibodies on cell functions or injury are some of the proposed mechanisms to explain the thrombotic tendency of patients with APS. Endothelial cells, monocytes and activated platelets may be a predominant target of aCL/β_2GPI associated with the pro-coagulant state characteristic of APS.

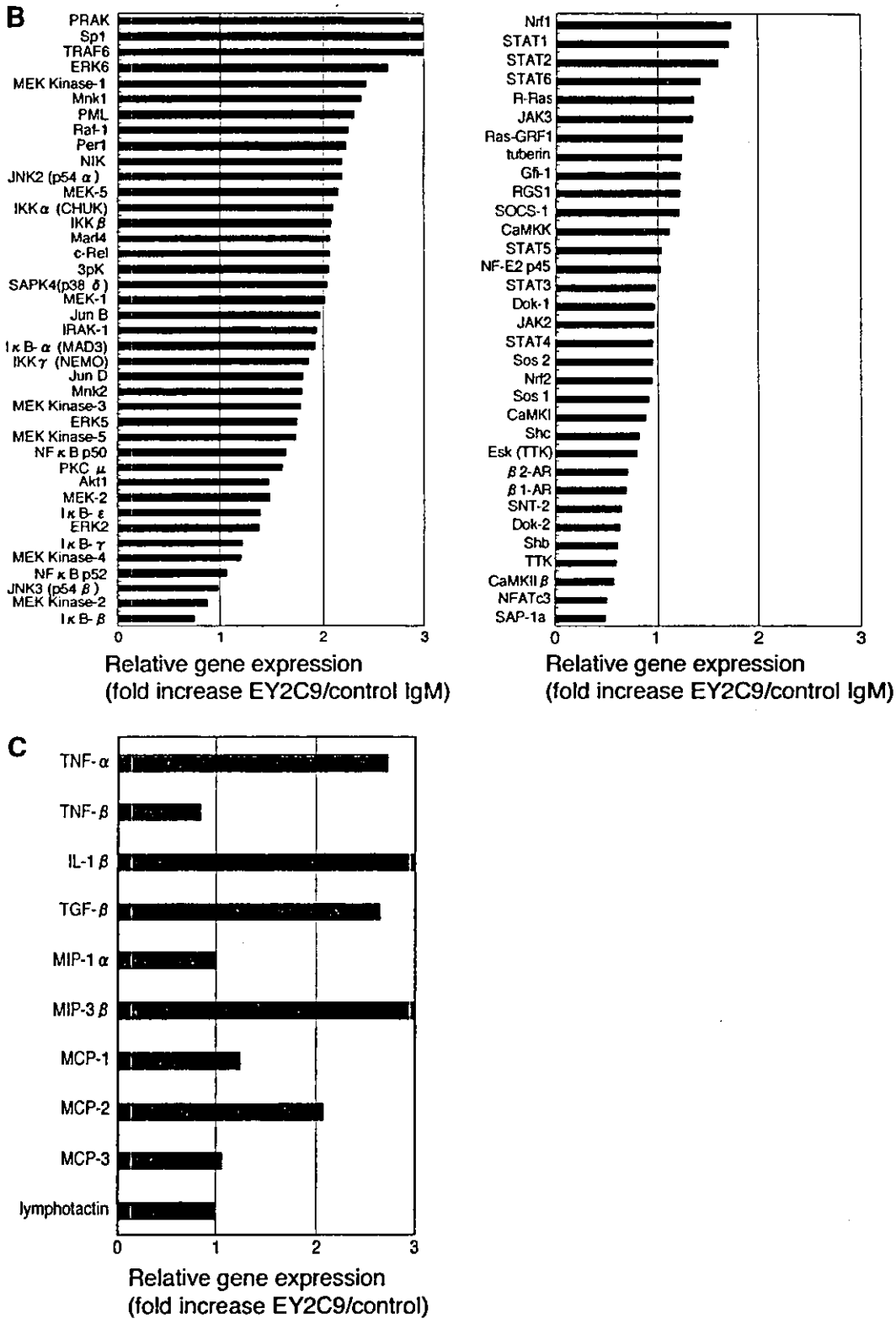

Fig. 1. *Continued.*

Pro-coagulant cell activation, accompanied with TF expression and TF pathway up-regulation, is one of the key events considered to explain the pathophysiology of thrombosis in patients with APS. We showed elevated plasma levels of soluble TF in APS patients (22, 35), and Cuadrado *et al.* (42) reported that monocytes prepared from APS patients had high

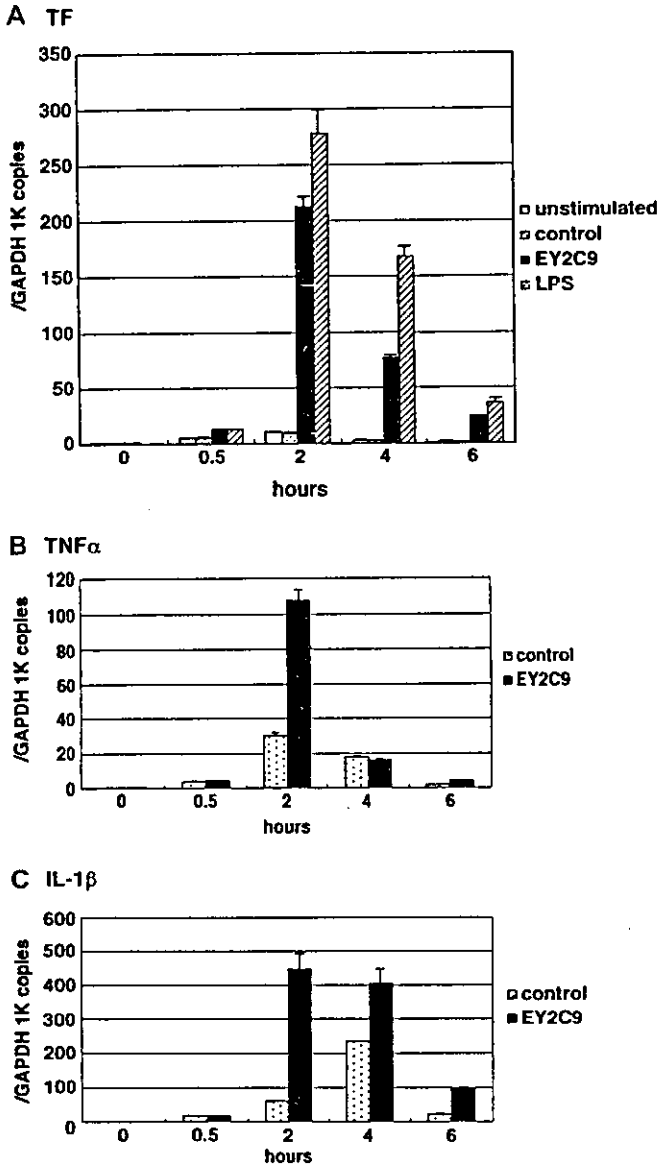

Fig. 2. Expression of TF, TNF-α and IL-1β mRNA in PBMCs treated with monoclonal aCL/β_2GPI. PBMCs were stimulated with 10 μg ml^{-1} monoclonal IgM aCL/β_2GPI (EY2C9 or TM1G2) or control IgM antibody (TM1B9) for 30 min and 2, 4 and 6 h. mRNA expression of TF (A), TNF-α (B) and IL-1β (C) were analyzed using real-time PCR as described in Methods. The maximum increase was observed at 2 h stimulation. Each column represents the average ± SD of three independent experiments.

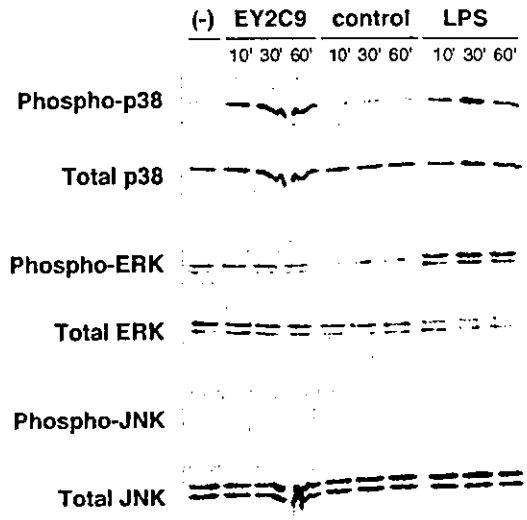

Fig. 3. MAPK phosphorylation in response to EY2C9. Mouse monocyte cell line RAW264.7 cells were stimulated with EY2C9 or control IgM antibody (TMIB9) for 10, 30 and 60 min. MAPK activation was determined by western blot using specific antibodies against total-p38, -ERK and -JNK/SAPK, and phospho-p38, -ERK and -JNK/SAPK.

TF expression. Tissue factor pathway inhibitor (TFPI), a physiological inhibitor of the extrinsic coagulation system, was also increased in plasma from patients with APS (22), suggesting up-regulation of TF and TFPI in affected patients. In *in vitro* experiments, numerous reports show that the IgG fraction from patients with aPL induced pro-coagulant activity on cells (38–41, 48). Our previous observation that human monoclonal aCL/β_2GPI induced TF mRNA and TF activity on PBMC or endothelium was confirmed by Reverter *et al.* (23) using the same mAbs. Apart from the TF molecule, other pro-coagulant substances induced by aPL were extensively investigated. Del Papa *et al.* (24, 49–51) have reported a series of molecules associated with endothelium activation by aPL *in vitro*, and other groups (25, 26) have shown adhesion molecules expression induced by IgG with aPL activity *in vitro* and *in vivo* models. Thus, it is widely accepted that aPL can induce the expression of TF or other pro-coagulant substances on cells in some conditions.

Recently, the signal transduction mechanism has been explored and associated with the increased expression of pro-coagulant substances in response to aPL. Dunoyer-Geindre *et al.* (52) presented an indirect but essential role of NF-κB in endothelial cell activation by aPL. IgG purified from APS patients induced the nuclear translocation of NF-κB leading to the transcription of a large number of genes that have a NF-κB-responsive element in their promoter. This nuclear translocation of NF-κB, at least in part, can explain the increased expression of TF by endothelial cell.

Protein kinases are key regulators of cellular signaling that control inflammation, cell differentiation, cell growth and cell death, and thus have been attractive targets for the treatment of neoplasms and inflammatory diseases. p38 MAPK was originally identified as a target molecule for a protein kinase inhibitor SB203580, a pyridinyl imidazole derivative which inhibits the production of pro-inflammatory cytokines. Isoforms of p38 MAPK are strongly activated by environmental stress or inflammatory cytokines [for review, see ref. (53)]. MAPK/ERK kinase (MEK)3 and MEK6, MAPKKs which obtain high specificity for p38, are activated by several MAPKKKs that become active by oxidative stress, ultraviolet irradiation, hypoxia, ischemia, Gram-negative bacteria-derived LPS (54, 55) or inflammatory cytokines such as TNF-α, IL-1β and IL-18. Accordingly, activation of p38 is considered to be critical for normal immune responses, and properties of the p38 pathway in inflammatory process have been investigated.