Fig. 5. Superimposed stereo view of domain IV structures of X-ray, IV–V interacted and 'triple' mutated models. Three domain IVs of the X-ray structure, the IV–V model and its triple mutation were superimposed using their main-chain atoms, and backbone smooth lines were colored blue, red and green, respectively. Each W^{235}, P^{187}, P^{189}, 193rd, 222nd and 228th residue was also described by the stick model in the same color scheme, with their residue names and numbers (only IV–V model) written in white. The side chains of P^{187} and P^{189} were close to the side chain of W^{235} by hydrophobic interaction.
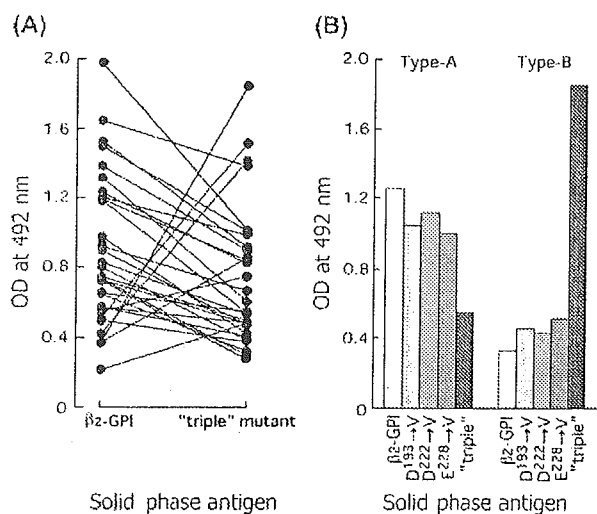

Fig. 6. Binding of antibodies to solid-phase mutant proteins. (A) Binding of antibodies in 30 anti-β2-GPI-positive APS serum samples to the 'triple' mutant protein was determined, as compared with that to control β2-GPI in anti-β2-GPI ELISA. (B) Binding of two typical antibodies (type A and type B) to mutant proteins was determined. β2-GPI and its mutant proteins were coated on a polyoxygenated plate and 100-fold diluted anti-β2-GPI antibody-positive sera were used.
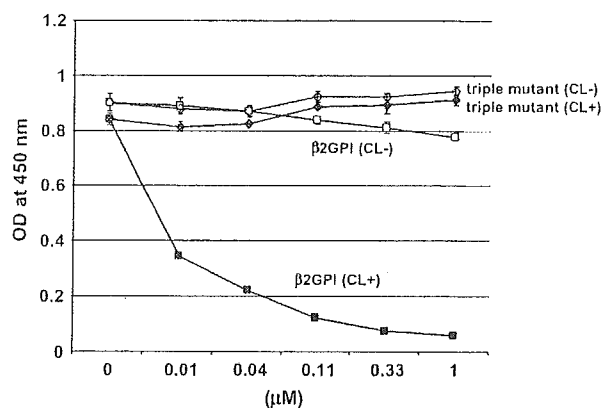

Fig. 7. Inhibition of the binding between WB-CAL-1 and β2-GPI coated on a polyoxygenated surface. β2-GPI was coated on polyoxygenated plates at a concentration of 10 μg ml^{-1} and the binding of WB-CAL-1 (1 μg ml^{-1}) was tested in the presence of inhibitors. The whole recombinant β2-GPI or the 'triple' mutant of β2-GPI was added as an inhibitor in the presence or absence of CL-liposome. Binding of WB-CAL-1 to the solid-phase β2-GPI was detected using HRP-conjugated anti-mouse IgG. Closed squares indicate β2-GPI with CL-liposome and open squares indicate β2-GPI without CL. Closed diamonds indicate triple mutant of β2-GPI with CL-liposome and open diamonds indicate the triple mutant of β2-GPI without CL.

Exposure of W^{235} by the inter-domain interaction between IV and V

Location of W^{235}, which was commonly found in all epitopic structures, was analyzed by making use of molecular modeling. As shown in Fig. 9, W^{235} partly located in an inner region in domain IV was exposed on the domain's outer surface by electrostatic interaction between domains IV and V, however, the residue was still hidden by domain V. Locations of

W^{235} and P^{189} in the model of nicked β2-GPI and in the triple-mutant model were similar to that in the X-ray structure. Nicked β2-GPI is a proteolytically cleaved form of β2-GPI that is generated mainly by plasmin *in vitro* and *in vivo* (28). This form of β2-GPI does not bind to PL surface and loses its function as a self-antigen (29). The analysis also showed that a structural
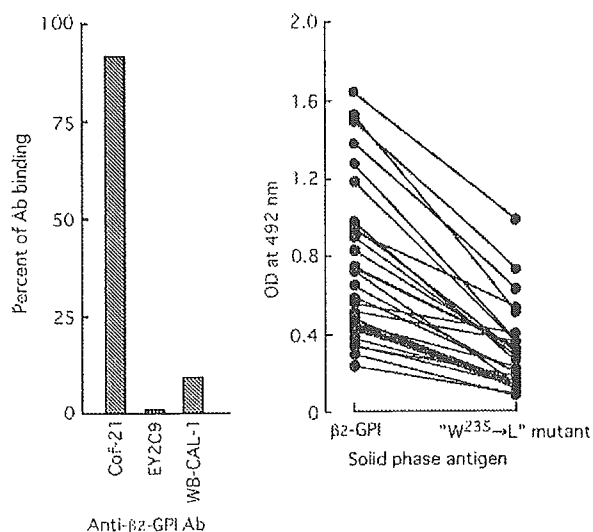
strain can occur by structural obstacles from the interactions of domain IV and V and from the location of the oligosaccharide attachment site (N^{234}) and it may result in the appearance of configurational changes in domain IV to expose the clusters to the epitopes.

Structural similarity to the region surrounding W^{235} residue in domain IV

Corresponding structures surrounding a tryptophan residue, i.e. W^{53}, W^{111}, W^{175} and W^{235} in domains I, II, III and IV, respectively, were compared (Fig. 10). The cryptic structure in domain IV was located on two discontinuous loops and a counterpart similar to the epitopic cluster was partly observed in domain I but amino acid composition was not fully complete. In domains II and III, some amino acid residues with different features were present in these counterparts for the epitopic cluster and the locations of the two loops were relatively different.

Fig. 8. Binding of anti-β2-GPI antibodies to solid-phase 'W^{235} → L' mutant protein. β2-GPI and its mutant proteins were coated on a polyoxygenated plate. Binding of mouse or human mAb (1 μg ml^{-1}) (A) and of antibodies in 30 anti-β2-GPI-positive APS sera to W^{235} → L mutant protein was determined. A 100-fold diluted anti-β2-GPI antibody-positive sera were used.

Discussion

β2-GPI was first described in 1961 to be a plasma protein (16) and complete amino acid sequences of human and bovine β2-GPI were determined by peptide sequencing (18, 19). Later, nucleotide sequence and deduced amino acid sequence of human β2-GPI were defined by cDNA cloning from human liver cells and by sequencing (30, 31). The secondary structure of β2-GPI was found to be composed of five homologous motifs, i.e. SCR/CCP repeats or sushi domains, which contain highly conserved half-cysteine residues, related to the formation of two internal disulfide bridges. The crystal structure of human β2-GPI has been reported (20, 21). In the present study, a model of the domain IV–V complex was
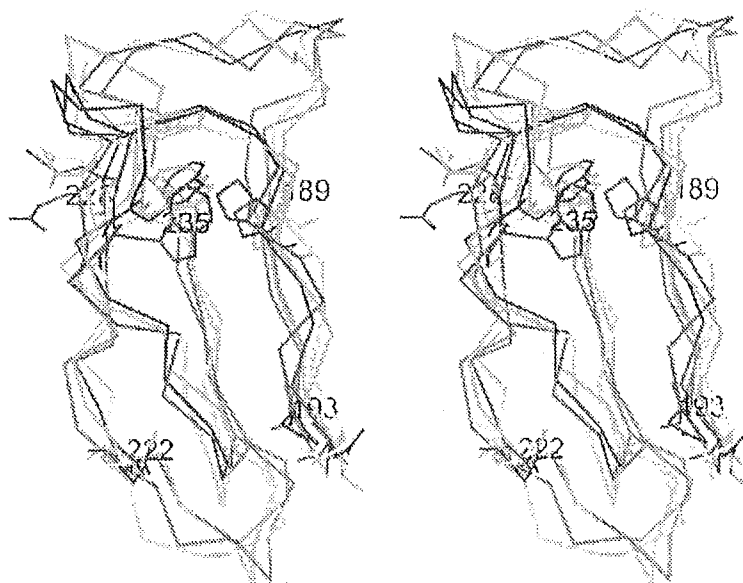

Fig. 9. Location of W^{235} predicted by molecular modeling. A stereoscopic superimposed view of Cα trace of domain IV in the X-ray structure (blue), the predicted domain IV–V model (red), the V-mutated model (green), or the nicked model (yellow) was shown with each structure of D (V)^{193}, D (V)^{222}, E (V)^{228}, W^{235} and P^{189}.
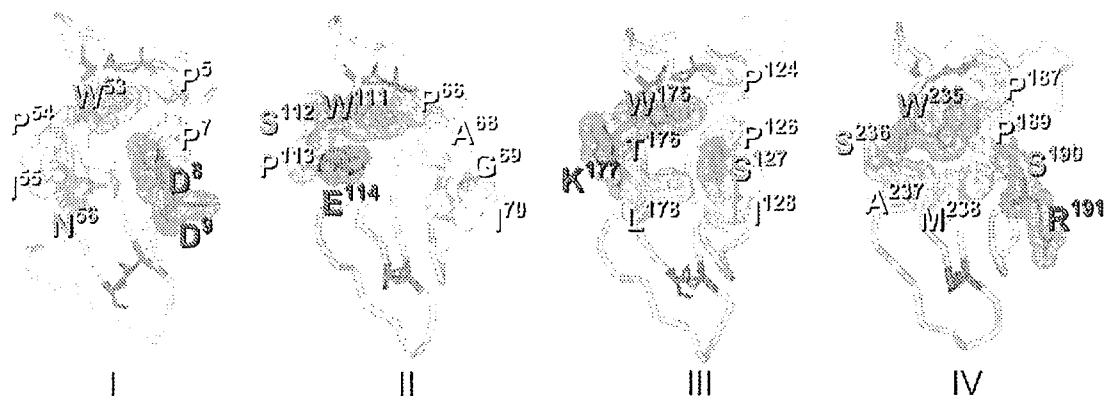
Fig. 10. Structural similarity of the corresponding region surrounding a tryptophan (W) residue in each domain. Cα traces of domains I, II, III and IV (of the domain IV–V model) in solvent phase are shown as white curves and with disulfide bridges (violet). Amino acid residues which commonly appeared on at least three out of four epitopic structures (for E1YC8, E1Y2C9, TM1G2 and WB-CAL-1) were superimposed on domain IV. Residues on domains I, II and III corresponding with the epitopic residues are also indicated in each domain, respectively. Positive-charged, negative-charged, non-charged hydrophilic and hydrophobic residues are in blue, red, yellow and green, respectively.

constructed by considering the crypticity of epitopic structures recognized by the auto-antibodies, as based on the elongated crystal structure of β2-GPI, and three inter-domain electrostatic interactions were observed in the optimized model (Fig. 3).

aCL bound to the solid-phase CL only in the presence of a plasma co-factor, i.e. β2-GPI, and the binding was not prevented in the excessive presence of fluid phase β2-GPI (8, 9). We also reported that such anti-β2-GPI auto-antibodies could bind to β2-GPI adsorbed on a polyoxygenated but not on a plain plate (10). Use of deletion mutant proteins indicated that domain IV was dominantly involved in expression of such epitopes and exposure of the epitopes was attributed to lack of domain V (11). All these data led to the hypothesis that such epitopes might be cryptic and locate on a hidden side of domain IV. We identified the structure of the cryptic epitopes recognized by human and mouse anti-β2-GPI auto-antibodies by making use of epitope mapping and phage libraries in the domain IV structure optimized from its crystal structure. In our β2-GPI model, all the antigenic amino acid clusters for the auto-antibodies mainly consisted of hydrophobic amino acids and were located on two discontinuous sequences in domain IV (Fig. 2). We also noted that it was able to construct a stable conformation of the domain IV–V complex, in which these regions of domain IV were partly on the inner side of the complex and were structurally hidden by overlying domain V (Fig. 3). In contrast, all epitopes specific for non-pathogenic mouse anti-human β2-GPI mAbs, such as Cof-18, Cof-20 and Cof-21, are located on the outer side of individual domains (Fig. 1).

The amino acid W^{235} was non-exceptionally utilized to form those antigenic structures for anti-β2-GPI auto-antibodies (Fig. 2). As shown in Fig. 7, L replacement of W^{235} significantly reduced antigenicity for either EY2C9, WB-CAL-1, and antibodies in 30 tested anti-β2-GPI antibody-positive serum samples. Thus, cryptic epitopes in domain IV might be recognized by the major population of anti-β2-GPI antibodies derived from APS and epitope spreading may occur around

the W residue. This hypothesis is also supported by the data in the study that replacement of a single amino acid at position 247, which is important for the interaction between domain IV and V, can alter the antigenicity of β2-GPI for pathogenic auto-antibodies, and also can be a risk factor for APS (32, 33).

In contrast, other research groups reported the presence of anti-β2-GPI auto-antibodies directed to domain V (12) or domain I (13), indicating that anti-β2-GPI auto-antibodies still seem to be heterogeneous. In 1996, we first indicated another possible epitopic location of domain I as well as domain IV (11) and as shown in Fig. 10, a similar structure to epitopic clusters located in domain IV was also found in domain I, albeit not fully complete. So, such auto-antibodies from APS may cross-react with the mimic structure in domain I but exact characterization will need to be done. According to the study done by de Laat et al. (34), two of three mutant β2-GPI which bear single amino acid mutation in domain I had reduced reactivity to IgGs from APS patients, indicating the importance of domain I for the binding of anti-β2-GPI antibodies. According to our inhibition ELISA, the mutation of three amino acids which are predicted to be critical for the interaction between domain IV and V of β2-GPI completely lost the inhibitory function found in native β2-GPI in the presence of CL–liposome. This result implies that exposure of the epitopes induced by the domain IV–V interaction in the presence of a negatively charged surface is critical for the binding of WB-CAL-1 (Fig. 7). Discrepancy in the results from these two different studies may indicate the heterogeneity of anti-β2-GPI antibodies, or structural similarity between the candidative epitopes predicted in domain I and IV (Fig. 10).

Cryptic epitopes that appear in the structure of domain IV were conformationally or configurationally altered by PL binding via the particular binding patch in domain V. The key amino acid residue W^{235} at the epitopic center was located in the inner region in domain IV of the crystal structure of the nicked β2-GPI model, and the β2-GPI model of the three V-replaced the β2-GPI model; however, we considered that the residue was exposed to the outer surface of the domain IV

molecule via the three electrostatic interactions. Further, a structural strain possibly occurs by PL binding to the patch which may result in configurational changes in domain IV via the structural obstacles from the domain IV–V interaction and from the oligosaccharide attachment site N^{234}.

The artificial triple mutation on of three specific electrostatic interactions between domains IV and V resulted in reduced antibody binding to the antigenic protein β2-GPI (Figs 3, 6 and 7). Thus, the constructed β2-GPI model and results from binding experiments indicated that domain V interacts with domain IV via these three specific electrostatic interactions and that these interactions are essential for expression of a group of cryptic epitopes in domain IV. These three electrostatic interactions between domain IV and V could contribute to the encrypting by covering W^{235}, the center residue of cryptic epitopes on domain IV, from the solvent phase and auto-antibody binding. Breaking of these interactions by mutations cause the de-encrypting by exposing the W^{235} residue to the solvent phase, and cryptic epitopes with W^{235} would be placed to the positions where they can bind to auto-antibodies. In contrast, our present study also indicated that there is another novel group of antigenic structures recognized by anti-β2-GPI auto-antibodies, which appeared only by the triple mutation in these *in vitro* systems (Fig. 6). Thus, cryptic epitopes which locate in domain IV would also be heterogeneous and at least two kinds of antibody populations would be present in APS patients. We entertained the notion that both populations of antibodies may be in close proximity with domain V yet be hidden. These electrostatic interactions linking domains IV and V may regulate the appearance of both types of cryptic epitopes closely located in domain IV. The core of the lysine-rich region of domain V seems to be constructed by K^{282}, K^{284}, K^{286}, K^{287}, K^{308} and K^{317} because of density of lysine on the molecular surface of the X-ray structure. By assuming that this core region could be strongly interacting with anionic PLs, K^{246}, K^{250} and K^{305} would not be affected by the binding of PLs, and it is also suggested that domain V combines with domain IV by the contact surface different from PLs.

In addition, a solution structure of this protein was reported by small-angle X-ray scattering (35) that the total conformation would be S shaped and had no direct interaction between domains IV and V. It was reliable as a total shape in the experimental solution. However, some oligosaccharide chains had been employed to compensate the moieties which could not be constructed by protein atoms, although each chain length was not measured correctly in the report.

In contrast, our model had been considered no oligosaccharide chain. The attachment of an oligosaccharide chain to the N^{234} side chain would not disturb the overlying of domain IV by domain V because the N^{234} side chain could have the opposite direction to the core of the cryptic epitope, the W^{235} side chain, on the same β-sheet conformation. It is necessary to get more information on the oligosaccharide chains to obtain the accurate structure.

It is absolutely true that dimerization of the protein increases the avidity to antibodies but the possibility that the interaction between PL and the protein creates a neoepitope also remains. Critical observation to support the latter idea is that even the triple mutant had a similar PL-binding property, and

the auto-antibody did not bind the mutant protein in the presence of PL. Thus, the interaction model between domains IV–V may be essential to expose the suitable structure for antibody binding. Antigenic structures which are recognized by different pathogenic anti-β2-GPI antibodies are not identical, even though they were found to be in a particular region on the inner side of domain IV of the β2-GPI molecule. Such responsiveness of antibodies to related multiple epitopes may indicate that intra-molecular epitope spreading may have occurred following initiation by a single epitope on the β2-GPI molecule. Although the majority of clinical manifestations associated with anti-β2-GPI antibodies is vascular occlusions by thrombosis either in vein or in arteries, symptomatic heterogeneity still exists in this syndrome. Establishment of clinical manifestations may depend on epitope spreading and specificity of anti-β2-GPI antibodies in each patient. The occurrence of epitope spreading and whether it contributes significantly to disease pathogenesis is potentially a key issue in designing antigen-directed therapeutics. Epitope mapping and the protein model we used may lead to identification of critical elements related to disease progression.

Acknowledgements

Abbreviations

aCLs	anti-cardiolipin antibodies
APS	anti-phospholipid syndrome
β2-GPI	β2-glycoprotein I
CCP	complement control protein repeat
CL	cardiolipin
PL	phospholipid
r.m.s.	root mean square
SCR	short consensus repeat
TBS	Tris-buffered saline

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REVIEW

Pathogenesis of antiphospholipid antibodies: impairment of fibrinolysis and monocyte activation via the p38 mitogen-activated protein kinase pathway

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Abstract

Antiphospholipid syndrome (APS) is characterized by recurrent thrombosis or pregnancy morbidity associated with antiphospholipid antibodies (aPL). Impaired fibrinolysis is a contributing factor for the development of thrombosis, and the effect of aPL in the fibrinolytic system has been investigated. Impaired release of tPA and enhanced release of PAI-1 after endothelial activation is reported in patients with APS. Elevated Lipoprotein (a) levels have been found in APS, which results in inhibition of fibrinolytic activity. Phospholipid-bound β_2-glycoprotein I (β_2GPI) is a major autoantigen for aPLs. β_2GPI exerts both anti-coagulant and pro-coagulant properties mainly by interacting with other phospholipid-binding proteins such as coagulation factors and protein C. Dramatic increase in the affinity of β_2GPI to the cell surface is induced by binding of pathogenic anti-β_2GPI antibodies, which may modify the physiological function of β_2GPI and may affect the coagulation/fibrinolysis balance on the cell surface. Using chromogenic assays for measuring fibrinolytic activity, we demonstrated that addition of monoclonal anticardiolipin antibody (aCL) decreases the activity of extrinsic/intrinsic fibrinolysis. Significantly lower activity of intrinsic fibrinolysis was also demonstrated in the euglobulin fractions from APS patients.

Endothelial cells and monocytes are activated by aPLs in vitro, resulting in production of tissue factor (TF), a major initiator of the coagulation system. Recently, aPLs are reported to induce thrombocytes to produce thromboxane. The importance of apoE receptor 2 on platelets for the binding of artificially dimerized β_2GPI was suggested. By investigating aPL-inducible genes in peripheral blood mononuclear cells, we found that the mitogen-activated protein kinase (MAPK) pathway was up-regulated. Using a monocyte cell line, phosphorylation of p38 MAPK, NF-κB translocation to the nuclear fraction, and up-regulated TF mRNA expression were demonstrated after treatment with monoclonal aCL. These phenomena were observed only in the presence of β_2GPI. Moreover, a specific p38 MAPK inihibitor SB203580 decreased aCL/β_2GPI-induced TF mRNA expression.

Thus, aCL/β_2GPI plays dual roles in the pathogenesis of APS, firstly by deranging the fibrinolytic system and secondly by activating monocytes, endothelial cells and thrombocytes to produce TF or thromboxane.

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Contents

Introduction

Antiphospholipid syndrome (APS) is a clinical condition characterized by recurrent arterial/venous thrombosis or pregnancy morbidity associated with antiphospholipid antibodies (aPL). These autoantibodies are not only markers of APS, but are also believed to play pathogenic roles in the development of symptoms in patients with APS (Pierangeli et al., 1996; Tsutsumi et al., 1996). The mechanism of aPL-induced thrombosis is not fully understood, although many discoveries have been made in these two decades. Impairment of fibrinolysis or acceleration of the coagulation system induced by aPL has been studied as the "classic" mechanism. Then, importance of premature atherosclerosis accelerated by aPL has been demonstrated in vitro and in vivo (George et al., 2000; Matsuura et al., 2002). Recently, activation of endothelial cells or other cell types has been focused on by many investigators. In addition, importance of complement system activation in pregnancy morbidity is also reported (Girardi et al., 2003). Thus, aPLs presumably induce thrombosis or pregnancy morbodity via multiple mechanisms. In this review, we focus on impairment of fibrinolysis and cell activation which are induced by aPL.

Impaired fibrinolysis

Impaired fibrinolysis is a contributing factor for the development of thrombosis, and the effect of aPL in the fibrinolytic system has been investigated. In patients with connective tissue diseases including APS, plasminogen activator inhibitor-1 (PAI-1) release after endothelial activation was greatly enhanced compared with healthy controls, but no difference was found in tissue plasminogen activator (tPA) release (Jurado et al., 1992). Impaired release of tPA and enhanced release of PAI-1 after endothelial activation suggests that tPA/PAI-1 balance is important in the development of thrombosis in APS (Ames et al., 1996). Elevated levels of lipoprotein (a) [Lp(a)] have been reported in APS patients (Atsumi et al., 1998). Lp(a) contains different numbers of kringle domains that interact with fibrinogen and inhibits fibrinolytic activity by inhibiting tPA

uncompetitively. Lp(a) also increases PAI-1 expression in endothelial cells.

In patients with APS, pathogenic aPL are not directed against phospholipid itself, but against phospholipid-binding proteins, such as β_2-glycoprotein I (β_2GPI), prothrombin, annexin V, protein C or protein S. Among these, phospholipid-bound β_2GPI is one of the major target antigens for aPLs present in patients with APS (McNeil et al., 1990; Galli et al., 1990; Matsuura et al., 1990). 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, and has been recognized as a natural anti-coagulant because β_2GPI inhibits prothrombinase and tenase function, factor XII activation and ADP-dependent activation of platelets (Nimpf et al., 1986, 1985; Schousboe and Rasmussen, 1995). Recently, β_2GPI has been shown to bind directly to factor XI and attenuate its activation (Shi et al., 2004). However, individuals with β_2GPI deficiency do not have a thrombotic tendency, thus anticardiolipin antibody (aCL)/β_2GPI-associated thrombosis cannot be merely explained by "β_2GPI insufficiency" (Yasuda et al., 2000; Takeuchi et al., 2000). β_2GPI also exerts pro-coagulant activities mainly by inhibition of protein C pathway (Mori et al., 1996). Binding of pathogenic anti-β_2GPI antibodies increases the affinity of β_2GPI to the cell surface (Takeya et al., 1997). Increased affinity of β_2GPI to the membrane may modify physiological function of β_2GPI and may affect the coagulation/fibrinolysis balance on the cell surface by interacting with other phospholipids-binding proteins such as coagulation factors and protein C. Using a chromogenic assay for measurement of extrinsic fibrinolysis, we demonstrated that addition of monoclonal aCL decreases the activity of extrinsic fibrinolysis in the presence of tPA, plasminogen, fibrin and β_2GPI (Ieko et al., 2000). We also demonstrated that addition of monoclonal aCL in the presence of β_2GPI decreased fibrinolytic activity by a newly developed chromogenic assay for measuring intrinsic fibrinolysis. In this system, in the presence of phospholipid and plasminogen, kaolin was added as a stimulator and plasmin generation was measured using plasmin specific substrate S-2251. When we measured intrinsic fibrinolysis activity of euglobulin fractions from APS patients and healthy controls, significantly lower activity of intrinsic fibrinolysis was evident in the patient group (Takeuchi et al.,
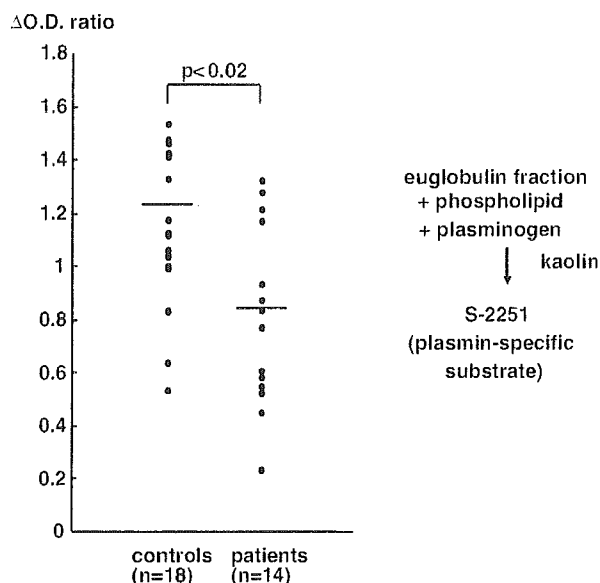
ΔO.D. ratio

Fig. 1. Activity of intrinsic fibrinolysis in APS patients. The intrinsic fibrinolytic activities of euglobulin fractions from APS patients and healthy controls were measured in the presence of β_2GPI, using kaolin as an activator (Takeuchi et al., 2000, 2002).

2002) (Fig. 1). These data suggest that impairment of intrinsic and extrinsic fibrinolysis induced by pathogenic anti-β_2GPI antibodies is one of the mechanisms for thrombosis in patients with APS.

Cell-activation mechanisms – contribution of p38 MAPK pathway

Recently, cell-mediated mechanisms have been reported in the formation of thrombosis in patients with APS. Endothelium is one of the major organs that cover the inner surface of blood vessels, and its perturbation or damage has been reported in many disorders. Inappropriately activated endothelial cells alter their properties from "antithrombotic" to "pro-thrombotic", by producing pro-coagulant substances and allies of adhesion molecules such as VCAM-1, ICAM-1, E-selectin, or endothelin–1. Actually, aPL induce tissue factor (TF) expression on endothelial cells in vitro, which results in the initiation of the extrinsic coagulation system (Amengual et al., 1998; Branch and Rodgers, 1993; Kornberg et al., 1994; Conti et al., 2003). TF is a cofactor for factor VIIa that activates factor IX and factor X. Then the factors IXa/Xa complex activates prothrombin to thrombin. Using endothelial cells, Raschi et al. (2003) reported the importance of TRAF6 and MyD88 in NF-κB activation induced by monoclonal aCL in the presence of β_2GPI.

They proposed that aCL reacts with β_2GPI likely associated to a member of the toll-like receptor/IL-1 receptor family. Annexin A2, alternatively named as annexin II, also mediates the binding of aCL/β_2GPI to the surface of endothelial cells (Ma et al., 2000; Zhang and McCrae, 2005). Annexin A2 is expressed on the endothelial cell surface and binds to β_2GPI with high affinity. They suggested that cross-linking of the cell-surface annexin A2 via aCL/β_2GPI stimulates activation of endothelial cells. However, how annexin A2 cross-linking mediates signal transduction remains unknown. Toll-like receptor is a candidate as a member of a multiprotein-signaling complex on the cell surface. Procoagulant activity of monocytes was reported to be increased in patients with systemic lupus erythematosus, although correlation with positive lupus anti-coagulant was not found (de Prost et al., 1990). Such procoagulant activities/TF expression in normal monocytes were induced by purified IgG from APS patients or aPL (Martini et al., 1996). We and others demonstrated the up-regulation of TF pathway in patients with APS (Atsumi et al., 1997; Cuadrado et al., 1997). Auto-antibodies against tissue factor pathway inhibitor (TFPI), which is a Kunitz-type protease inhibitor that inhibits TF activity by forming a complex with TF, factor VIIa and Xa, have been detected in APS patients (Forastiero et al., 2003). Thus, cell-mediated TF up-regulation and antibody-mediated TFPI down-regulation work coordinately toward the hyper-coagulable state. Recently, aPL have been reported to induce thrombocytes to produce thromboxane in the presence of subactivating amount of thrombin. Platelets sensitized by aCL/β_2GPI showed increased deposition on a collagen-containing surface. In this study, interaction between dimerized β_2GPI and apolipoprotein E receptor 2 (ApoER2) on platelets was reported (Lutters et al., 2003). These findings partly explain the pathophysiology in this syndrome.

In order to address the question how the binding of aPL/cofactor to these cell surfaces causes production of pro-coagulant molecules, we investigated aPL-inducible genes in peripheral blood mononuclear cells using a cDNA array system. Two hours after exposure to EY2C9, a monoclonal IgM class aCL established from an APS patient, mRNAs related to the mitogen-activated protein kinase (MAPK) pathway, such as p38-regulated/activated protein kinase (PRAK), Sp-1, TNF receptor-associated factor 6 (TRAF6) and SAPK4 (p38δ), were increased more than two-fold. TF and inflammatory cytokines such as TNF-α and IL-1 expression were also confirmed using real-time PCR (Bohgaki et al., 2004). Using monocyte cell line RAW264.7, phosphorylation of p38 MAPK, translocation of NF-κB to the nuclear fraction, and expression of TF mRNA were demonstrated after treatment with monoclonal aCL. These phenomena were observed only

in the presence of β_2GPI. Moreover, a specific p38 MAPK inihibitor SB203580 decreased aCL/β_2GPI-induced TF mRNA expression.

Almost simultaneously, Vega-Ostertag et al. (2004) treated platelets with aPL and demonstrated increased phosphorylation of p38 MAPK and production of thromboxane B2, which was abrogated by SB203580. F(ab') fragments of purified IgG from patients effectively increased the phosphorylation of p38 MAPK and calcium-dependent cytosolic phospholipase A$_2$, but not that of ERK-1/2 MAPKs. Recently, the same group investigated human umbilical endothelial cells (HU-VECs) in a similar system and have demonstrated the involvement of p38 MAPK in the up-regulation of TF (Vega-Ostertag et al., 2005). This up-regulation was again inhibited by SB203580, and also by MG132, a specific inhibitor for the downstream NF-κB. They also found that aPL induced HUVECs to express IL-6, IL-8, and inducible nitric oxide synthase, and that these processes involve p38 MAPK activation. Thus, the p38 MAPK pathway plays an important role in the aPL-mediated activation of endothelial cells, monocytes, and

platelets, providing a possible therapeutic target in APS. p38 MAPK isoforms are activated by environmental stress such as oxidative stress, UV irradiation, hypoxia, ischemia, Gram-negative bacteria-derived LPS, or inflammatory cytokines such as TNF-α, IL-1β, and IL-18. Activation of p38 MAPK induces proinflammatory cytokines, such as TNF-α and IL-1β resulting in enhancement of inflammatory reaction. Following p38 MAPK phosphorylation, transcriptional factors such as activating transcriptional factor-2 (ATF2) are activated, which form a heterodimer with Jun family transcriptional factors and associates with the activator protein-1 (AP-1) binding site. NH$_2$-termini of histone H3 undergoes structural alteration in a p38-dependent pathway after LPS stimulation, which results in enhancement of accessibility of the cryptic NF-κB binding sites (Saccani et al., 2002). The promoter region of the TF gene contains two AP-1 binding sites and one NF-κB binding site, and these transcription factors are proven required for maximal induction of TF gene transcription. Proposed mechanisms of cell activation induced by aPL are illustrated in Fig. 2.
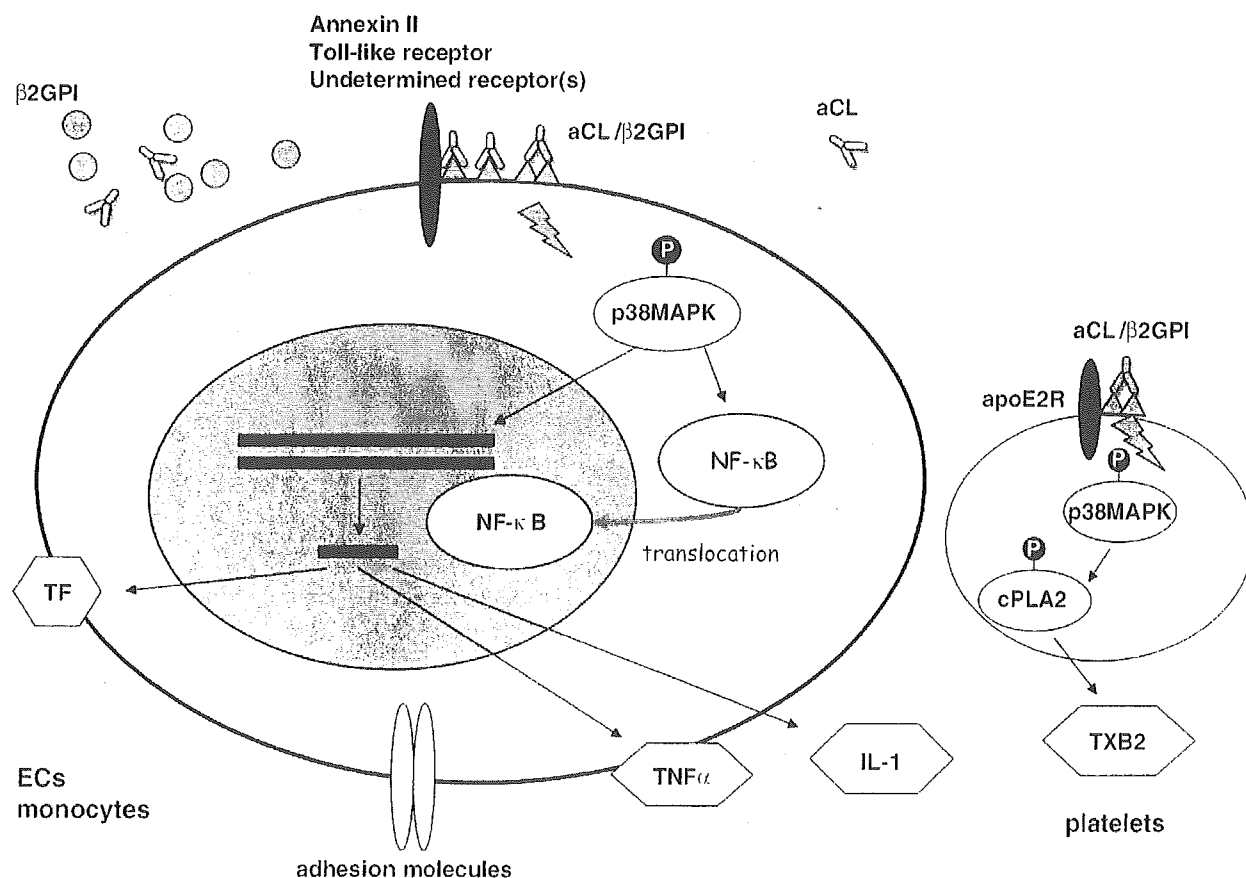

Fig. 2. Proposed function of antiphospholipid antibodies on endothelial cells, monocytes, or platelets. β_2GPI, β_2-glycoprotein I; aCL, anticardiolipin antibody; p, phosphorylation; EC, endothelial cell; TF, tissue factor; ApoE2R, apolipoprotein E receptor 2; cPLA$_2$, calcium-dependent cytosolic phospholipae A$_2$; TXB$_2$, thromboxane B$_2$.

Thus, it would be reasonable as a choice for the treatment of APS, to suppress p38 MAPK activation using its inhibitors. In murine models, administration of SB203580 was beneficial for endotoxin-induced shock and collagen-induced arthritis. Several other inhibitors for p38 MAPK have been developed and some of these inhibitors were tested in clinical trials. For example, BIRB796 inhibited LPS-induced coagulation activation, as measured by plasma concentrations of the prothrombin fragment F1+2, during human endotoxemia (Branger et al., 2003). RWJ67657 inhibited TNF-α, IL-8, and IL-6 in human without significant adverse effects (Faas et al., 2002). Although expression of p38 MAPK is relatively ubiquitous and p38 MAPK also activates anti-inflammatory IL-10 and tumor-suppressive p53, p38 MAPK suppression is an attractive choice of treatment in the future. At the same time, hunting for more specific treatment targets would be favorable. The mechanisms are not fully understood how aCL/β_2GPI binds to the cell surface and how signal transduction events occur upstream of p38 MAPK in monocytes, which are a major producer of TF, although ApoER2 on platelets and toll-like receptor or annexin II on endothelial cells are proposed as "ligands" for β_2GPI.

Conclusion

aCL/β_2GPI plays multiple roles in the pathogenesis of thromboses found in APS, firstly by deranging the fibrinolytic system, secondly by accelerating atherosclerosis, and lastly by activating monocytes, endothelial cells and thrombocytes to produce TF or thromboxane. To date, anti-platelet agents or anti-coagulants are utilized to prevent the recurrence of thrombosis in patients with APS. However, because of the difference of bioavailability among patients or of adverse effects, more specific therapy would be desirable. Understanding the interaction between aPL and cell surface and following signaling events will provide tools for developing novel therapies in patients with APS.

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CASE REPORT

Acute inflammatory sensorimotor polyradiculoneuropathy associated with immune thrombocytopenic purpura

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Abstract. Sato N, Kamata T, Akiyama N, Kuwana M, Kanda T (Tokyo Metropolitan Bokutoh Hospital, Tokyo; Keio University School of Medicine, Tokyo; and Yamaguchi University School of Medicine, Ube; Japan). Acute inflammatory sensorimotor polyradiculoneuropathy associated with immune thrombocytopenic purpura (Case Report). *J Intern Med* 2005; 257: 473–477.

Although acute inflammatory polyneuropathy (AIP) and immune thrombocytopenic purpura (ITP) are both believed to be immune-mediated disorders, only a few cases have been reported in which these two diseases co-existed. We describe a case of a 67-year-old patient who developed quadriparesis, ophthalmoplegia and severe sensory impairment along with thrombocytopenia. Detailed examinations, including the measurement of anti-ganglioside antibodies and anti-glycoprotein-IIb-IIIa-IgG-producing B-cells, revealed that he developed AIP and ITP. By reviewing past similar reports, we noticed that AIP associated with ITP tends to manifest severe sensory impairment and is often preceded by upper respiratory tract infection, but not by gastrointestinal infection.

Keywords: acute inflammatory polyneuropathy, anti-ganglioside antibody, anti-GD1b antibody, glycoprotein IIb-IIIa, immune thrombocytopenic purpura.

Introduction

It is uncommon that peripheral neuropathy is observed in association with immune thrombocytopenic purpura (ITP). Amongst such cases, peripheral neuropathy is sometimes described to be mononeuropathy multiplex and is attributed to intraneural haemorrhage [1]. Acute inflammatory polyneuropathy (AIP), on the contrary, is rarely reported in relation with ITP, although they are both believed to be immune-mediated disorders. Most of the reports of AIP associated with ITP merely described the clinical findings of individual cases, and immunological investigations, such as the measurement of anti-ganglioside antibodies, are far from satisfactory. Here we describe a patient in whom acute inflammatory sensorimotor polyradiculoneuropathy (AISMP) and ITP developed simultaneously, and by reviewing past similar reports, discuss the characteristics of AIP associated with ITP.

Case report

A previously healthy 67-year-old man with 4-day history of a low-grade fever and a sore throat developed tingling feelings in the distal parts of his extremities and came to our hospital at the end of June 2003. Neurological examination revealed wide-based gait and diminished deep tendon reflexes. His blood sample showed a markedly decreased platelet count.

The next morning, the platelet count was reduced to $2.0 \times 10^9 \, \mathrm{L}^{-1}$ and the peripheral blood smear revealed no morphological abnormalities. He had not taken any drugs except for mixed vitamin supplements, and bone marrow examination showed a normal megakaryocyte count and no dysplasia. A preliminary diagnosis of ITP was made and steroid pulse therapy (methylprednisone 1 g day^{-1} for 3 days) was carried out, which rapidly improved the platelet count. Although platelet-

associated IgG was not elevated, enzyme-linked immunospotting (ELISPOT) assay [2] revealed that the number of peripheral blood B-cells producing IgG anti-glycoprotein-IIb-IIIa (GPIIb-IIIa) antibodies was increased to 6.6 cells per 10^5 peripheral blood mononuclear cells (normal: <2.0). Oral prednisone was started with 50 mg day^{-1} following the steroid pulse therapy and the dose was gradually tapered off.

During the 5 days following his admission, his neurological conditions rapidly deteriorated. He developed quadriparesis, especially notable in the proximal muscles, so that he became unable to lift his limbs. The sensory disturbance was none the better. The vibration sense and proprioception were more severely impaired than the superficial sense. He also developed ophthalmoplegia, and bulbar palsy was also prominent. He required mechanical ventilation for 10 days. There were no symptoms suggesting autonomic involvement.

His cerebrospinal fluid specimen showed a normal cell count and an elevated level (1.892 g L^{-1}) of protein. An investigation into anti-ganglioside antibodies revealed an elevated level [102 400× (normal: not more than 6400×)] of anti-GD1b antibody (Ab), and a borderline increase in the level of anti-GQ1b Ab.

In order to eliminate other autoimmune diseases that would cause thrombocytopenia and/or neuropathy, screening tests for autoantibodies, including antinuclear Ab, anti-Ro/SS-A Ab, anti-La/SS-B Ab, anti-cardiolipin Ab, myeloperoxidase-anti-neutrophil-cytoplasmic Ab (MPO-ANCA) and proteiase-3-ANCA, were carried out, which gave negative results. The search for preceding infection was unsuccessful, but we excluded such infections as those of Epstein–Barr virus, cytomegalovirus, human immunodeficiency virus, hepatitis B virus and hepatitis C virus. No malignancy was found and anti-Hu Ab was not detected, and thus the presence of paraneoplastic polyneuropathy was unlikely.

The nerve conduction study revealed decreased motor conduction velocity and prolonged distal motor latency. Conduction block was observed in the left ulnar nerve and temporal dispersion was seen in the left peroneal nerve. Sensory nerve action potentials could not be detected in any of the tested nerves. Denervation potentials and decrease in the number of motor units were observed by needle

electromyogram performed later in the course. Sympathetic skin response, the coefficient of variance for R-R intervals of the electrocardiogram and myocardial ^{123}I-métaiodobenzylguanidine scintigraphy (which reflects the sympathetic innervation of the cardiac muscle) showed no abnormalities, which was consistent with the symptoms lacking autonomic involvement. Sural nerve biopsy performed 12 days after the disease onset demonstrated a number of swollen axons and undigested myelin ovoids without any findings of haemorrhage, vasculitis or inflammatory cell infiltration, i.e. a nonspecific finding of acute axonal degeneration.

Intravenous immunoglobulin therapy (0.4 g kg^{-1} body weight per day for 5 days) was given following the steroid pulse therapy. He was successfully weaned away from mechanical ventilation, and gradually able to move his eyes. Improvement was fairly apparent as for superficial sense, but not for deep sense. Muscle weakness improved to a certain degree, but he was still confined to a wheelchair and could not swallow food at the end of March 2004.

The platelet count started to decrease again in August, 2003. Prednisone was increased, but the response was not as good as the previous treatment, and the platelet count fluctuated in the range of $10.0–300 × 10^9$ L^{-1} since then.

Comment

We describe a case of AISMP that simultaneously developed with ITP following upper respiratory tract infection (URI). Proximal-dominant muscle weakness was fairly severe, but the sensory involvement, especially as with vibration and proprioception, was none the less prominent. Cranial nerve palsy that resulted in ophthalmoplegia, dysphagia and dysarthria was also apparent and there was no sign of damage to the autonomic nervous system.

Anti-GD1b Ab, which is known to sometimes appear in sensory ataxic form of Guillain–Barré syndrome (GBS) [3], was detected in the present case. As GD1b is abundantly expressed in the dorsal root ganglia (DRG) [4] and it is shown that rabbits immunized with GD1b develop sensory ataxic neuropathy [5], the severe impairment of deep sensation in the present patient might be due to anti-GD1b-Ab-mediated impairment of DRG. The slightly elevated level of anti-GQ1b Ab in the present case is somewhat difficult to interpret. It may be only the

Table 1 A summary of reported cases of acute inflammatory polyneuropathy associated with immune thrombocytopenic purpura, showing the present case at the bottom

References	Patient (age/sex)	Preceding infection	Neurological impairments	Cranial nerve involvement	Electrophysiological findings	BMA findings	Other laboratory findings	Treatment
Gross (1980) [16]	30/F	URI	Mot (prox) < Sens (D)	Bil N. VII + soft palatal palsy	CMAP: ↓MCV SEP: ND	↑Mgk		PSL
Fukayama et al. (1983) [14]	32/F	Rubella	Mot < Sens (D + S)		MCV: lower limit SNAP: ND	↑Mgk	↑Anti-rubella Ab Anti-platelet Ab(+)	PSL, IVIg, AZT
Combarros et al. (1991) [12]	75/F	URI	Mot (prox) > Sens	Rt N. VII + IX	CMAP: ↓MCV, ↓Amp SNAP: ↓SCV, ↓Amp F-wave: ND	↑Mgk	PA-IgG not elevated	PSL
Corbanese et al. (1998) [13]	50/F	URI	Mot > Sens	Bil N. VII + soft palatal palsy	CMAP: ↓MCV F-wave: ND	Not described	Campylobacter jejuni(−)	IVIg, mPSL
Khaldi et al. (1990) [17]	3/F	URI	Mot		Not described	Compatible with ITP		Not described
Vashista et al. (1992) [18]	50/F	Not depicted	Mot	Bil N. VII + IX	Not described	→Mgk	HIV(−) CMV(−), EBV(−)	PSL
Gaur et al. (2003) [15]	73/M	Not depicted	Mot		Compatible with demyelinating polyradiculoneuropathy	Not described	Direct Coombs(+)	IVIg
Present case	67/M	URI	Mot (prox) ≤ Sens (D + S)	Ophthalmoplegia + bulbar palsy	CMAP: ↓MCV, ↓Amp CB(+), TD(+) SNAP: ND	→Mgk	Anti-GD1b Ab(+) Anti-GQ1b Ab(+/−) ↑Anti-GPIIb-IIIa-IgG-producing B-cells	mPSL, PSL, IVIg

BMA, bone marrow aspiration; M, male; F, female; URI, upper respiratory infection; Mot, motor; prox, proximal-dominant; Sens, sensory; D, deep sense; S, superficial sense; >, more prominent than; <, less prominent than; ≤, not severer than; Bil, bilateral; N. VII, facial nerve palsy; N. IX, glossopharyngeal nerve palsy; CMAP, compound muscle action potentials; MCV, motor conduction velocity; Amp, amplitude; SNAP, sensory nerve action potentials; ND, not detected; SEP, somatosensory evoked potentials; CB, conduction block; TD, temporal dispersion; ↑, increased; →, normal number of; ↓, decreased; Mgk, megakaryocytes; Ab, antibody; PA-IgG, platelet-associated IgG; HIV, human immunodeficiency virus; CMV, cytomegalovirus; EBV, Epstein-Barr virus; (+), positive or detected; (−), not detected; (+/−), borderline increase in; PSL, prednisone; mPSL, methylprednisone pulse therapy; IVIg, intravenous immunoglobulin therapy; AZT, azathioprine.

manifestation of cross-reaction between anti-GD1b Ab and anti-GQ1b Ab [6], or may be related to the clinical symptoms that included Miller–Fisher syndrome-like aspects.

The thrombocytopenia in the present case was diagnosed as ITP based on the practice guideline published by the American Society of Hematology [7]. There was no history that would suggest other causes of thrombocytopenia, including drug-induced thrombocytopenia, the peripheral blood smear showed normal morphology, and the examination of the bone marrow revealed no abnormality. The diagnosis was further supported by the ELISPOT assay which showed an increase in the number of peripheral blood B-cells that produce anti-GPIIb-IIIa IgG. It is widely accepted that GPIIb-IIIa is the major target for the autoimmune response in ITP [8, 9], and there is a report that indicates this ELISPOT assay is valuable in the diagnosis of ITP with high sensitivity (71/78) and specificity (30/32) [2].

GPIIb-IIIa, a member of the integrin family, is a heterodimer consisting of α- and β-subunits ($\alpha_{IIb}\beta_3$). The α_{IIb}-subunit is specifically expressed in platelets and megakaryocytes [10], where as the β_3-subunit is more variously expressed in endothelial cells, placental syncytiotrophoblast brush border, osteoclasts and macrophages. There have been no reports that showed their expression in the nervous tissue so far. Conversely, the ganglioside constituents of platelets are known to be completely different from those of the nervous tissue [11]. Thus, it is not easy to show the presence of a common antigen that would explain the association of AIP and ITP, though possibility remains that they share a common target epitope.

To our knowledge, there have been only seven cases published so far in which AIP and ITP developed in same patients [12–18]. Their characteristics are summarized in Table 1 along with those of our case. Although the ages of the patients are diverse, six of eight cases are of women, and URI was the preceding infection in five out of eight, whereas diarrhoea was not documented in any cases. Three cases are reported to have manifested motor-dominant symptoms typical of GBS, whilst three, including the present case, developed severe sensory involvement, especially of the deep sense. There have been no cases in which autonomic impairment was observed. No previous reports described anti-ganglioside-Ab profile or proved autoimmune response to GPIIb-IIIa.

It could be said that the association of AIP and ITP is a mere coincidence, but AIP that develops with ITP seems to have the properties somewhat different from typical GBS, including frequent severe sensory involvement with rare autonomic manifestations and gastrointestinal infection not preceding the disease. Further investigations into similar cases are needed to clarify its characteristics.

Conflict of interest statement

No conflict of interest was declared.

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BRIEF COMMUNICATION

Interleukin-10 genotypes are associated with systemic sclerosis and influence disease-associated autoimmune responses

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Systemic sclerosis (SSc; scleroderma) is a connective tissue disease, characterized by fibrotic, immunological, and vascular abnormalities. Interleukin-10 (IL-10) is an anti-inflammatory cytokine that modulates collagen production and B-cell survival. To determine if certain IL-10 genotypes are risk factors for the development of SSc and influence disease-associated autoimmune responses, 248 Caucasian and 264 Japanese SSc patients and controls were genotyped for three loci: −3575, −2849, and −2763. Sera from patients were characterized for SSc-associated autoantibodies. In Caucasians, at −3575 and −2763, the frequency of AA homozygotes was higher in patients as compared with controls (P = 0.0005; P = 0.002). In Japanese subjects, the frequency of AC heterozygotes at −2763 was higher, and that of CC homozygotes lower, in patients with diffuse SSc as compared to controls (P = 0.04). Particular IL-10 genotypes were associated with SSc-related autoantibodies. These results suggest that IL-10 genotypes contribute to the etiology of scleroderma.
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Interleukin-10 (IL-10) is a pleiotropic cytokine produced primarily by monocytes, T cells, and B cells. IL-10 has both activating and inhibitory influences on T cells, is involved in immunoglobulin class switching, and promotes B-cell survival.[1] Additionally, IL-10 modulates the extracellular matrix by inhibiting fibroblast proliferation and collagen production.[2,3] The interindividual differences in IL-10 production levels have a large genetic component.[4–6]

Owing to these properties, there is a growing interest in determining the role of IL-10 genes in autoimmune diseases.[7] Scleroderma is an autoimmune rheumatic disease characterized by extensive fibrosis, thickened skin, vascular alterations, and immunological abnormalities. There are two major subtypes of systemic sclerosis (SSc).[8] Diffuse SSc is the most serious form, characterized by extensive fibrosis of skin and internal organs; development of the disease can be rapid and severe. Limited SSc is a milder disease form, as patients have less involvement of internal organs and slower disease progression. The majority of SSc patients produce antibodies directed at nuclear antigens, which are strongly associated with organ involvement and disease outcome.[9–12]

Although the etiology of SSc is unknown, genetic factors are thought to be important. Several polymorphic genes—which are either risk factors for the development of SSc or influence disease-associated autoimmune responsiveness—have been identified. These include human leukocyte antigens (HLA), fibrillin-1, and GM and KM allotypes—genetic markers of γ and κ chains, respectively.[13–15] To determine if allelic variation at IL-10 loci is associated with SSc and SSc-related humoral autoimmune responses, DNA samples from Caucasian and Japanese patients and controls were genotyped at three IL-10 sites (−3575, −2849, and −2763) and patients' sera were characterized for disease-related autoantibodies.

This study was approved by the Institutional Review Board/Ethical Committee for human research of the Medical University of South Carolina and the Keio University School of Medicine. All subjects provided their written informed consent. Caucasian subjects consisted of 105 SSc patients (67 limited, 38 diffuse) and 143 controls presenting at the Rheumatology Clinic of the Medical University of South Carolina. Controls consisted of unrelated patients with osteoarthritis, fibromyalgia, gout, or regional musculoskeletal pain syndromes. Controls with conditions associated with autoimmune or connective tissue diseases were excluded. Japanese subjects consisted of 127 SSc patients (86 limited, 41 diffuse) presenting at Keio University School of Medicine and 137 healthy, ethnically matched controls living in the Tokyo area. All patients fulfilled the

American College of Rheumatology criteria for SSc.[16] Genomic DNA samples were genotyped by PCR-restriction fragment length polymorphism methods. The method for genotyping at −3575 was modified from Moraes *et al*.[17] A 228 base pair (bp) sequence containing the −3575 single-nucleotide polymorphism (SNP) was amplified (F 5′-GGT TTT CCT TCA TTT GCA GC-3′ and R 5′-ACA CTG TGA GCT TCT TGA GG-3′) and digested with the restriction enzyme *Tsp509I*, which cuts this product into 121 and 107 bp pieces in the presence of the T allele. Genotypes at positions −2849 and −2763 were determined using the same primer set (F 5′-ACA TTT CAG AAC AAA TAA AGA AGT CAG-3′ and R 5′-GTG CAG TGG CAT GAT CTC AG-3′) to amplify a 300 bp product including both SNPs. Digestion with *AlwI* results in products of 202, 77, and 21 bp for the G allele, and products of 279 and 21 bp, if the A allele is present at position −2849. Digestion with *Tsp509I* results in products of 174 and 126 bp, if the A allele is present at position −2763; no digestion of the 300 bp amplified product occurs in the presence of the C allele. Indirect immunofluorescence (anticentromere (ACA)), double immunodiffusion and protein immunoprecipitation (anti-topoisomerase I (topo I)), and immunoprecipitation (anti-RNA polymerase I/III (RNAP) and U1 ribonucleoprotein (U1 RNP)) assays were used to identify autoantibodies in patients' sera, as described previously.[12]

The distribution of genotype frequencies was analyzed using Pearson's χ^2 test, except when cell counts were less than or equal to 5; in the latter case, data were analyzed by Fisher's two-tailed exact test. Statistical significance was defined as $P < 0.05$. Odds ratio (OR) was calculated to measure the strength of the associations observed. When cell counts were less than or equal to 5, no ORs were determined, as the use of large sample theory to calculate confidence intervals for the ORs can only be justified when all of the expected cell counts are greater than 5.

The distribution of IL-10 genotypes and prevalence of autoantibodies in Caucasian subjects is presented in Table 1. At −3575 and −2763, the frequency of AA homozygotes was significantly higher in patients as compared with controls (29.5 *vs* 11.8%, $P = 0.0005$, OR = 3.1, CI 1.6−5.9; 20.6 *vs* 7.2%, $P = 0.002$, OR = 3.3, CI 1.5−7.5). At position −2849, the frequency of GG homozygotes was significantly lower in patients as compared to controls (44.2 *vs* 57.4%, $P = 0.04$, OR = 0.59, CI 0.35−0.98). Subdivision of the patient population showed that subjects with the limited form of the disease were the primary contributors to the overall variation at −3575 and −2849. Compared to controls, the frequency of AA homozygotes (−3575) was increased (33 *vs* 11.8%, $P = 0.0002$, OR = 3.6, CI 1.76−7.43), while that of GG (−2849) was decreased (41.8 *vs* 57.4%, $P = 0.03$, OR = 0.53, CI 0.29−0.96) in patients with limited SSc. At −2763, however, subjects in both disease categories contributed to the overall variation. Compared to controls, the frequency of AA homozygotes was higher in both limited and diffuse SSc (21 *vs* 7.2%, $P = 0.003$, OR = 3.5, CI 1.48−8.48; 19 *vs* 7.2%, $P = 0.03$, OR = 3.0, CI 1.06−8.55). The frequency of the AC genotype at this locus was significantly lower in patients

Table 1 Distribution of IL-10 genotypes and prevalence of autoantibodies in Caucasian subjects

Subjects, N (%)	−3575 genotype			−2849 genotype			−2763 genotype		
	AA	AT	TT	AA	AG	GG	AA	AC	CC
Total SSc	31 (29.5)[a]	39 (37.1)	35 (33.3)	10 (9.6)	48 (46.2)	46 (44.2)[b]	21 (20.6)[c]	39 (38.2)	42 (41.2)
RNAP+	2 (10.0)	6 (30.0)	12 (60.0)[d]	1 (5.0)	6 (30.0)	13 (65.0)[e]	2 (10.0)	4 (20.0)	14 (70.0)[f]
RNAP−	29 (34.1)	33 (38.8)	23 (27.1)	9 (10.7)	42 (50.0)	33 (39.3)	19 (23.2)	35 (42.7)	28 (34.1)
U1 RNP+	4 (40.0)	4 (40.0)	2 (20.0)	2 (22.2)	5 (55.6)	2 (22.2)	4 (40.0)	5 (50.0)	1 (10.0)[g]
U1 RNP−	27 (28.4)	35 (36.8)	33 (34.7)	8 (8.4)	43 (45.3)	44 (46.3)	17 (18.5)	34 (37)	41 (44.5)
Diffuse SSc	9 (23.7)	15 (39.5)	14 (36.8)	3 (8.0)	16 (43.0)	18 (48.7)	7 (19.0)[h]	10 (27.5)[i]	20 (54.0)
RNAP+	2 (11.8)	5 (29.4)	10 (58.8)[j]	1 (5.9)	5 (29.4)	11 (64.7)	2 (11.8)	3 (17.6)	12 (70.6)
RNAP−	7 (33.3)	10 (47.6)	4 (19.0)	2 (10.0)	11 (55.0)	7 (35.0)	5 (25.0)	7 (35.0)	8 (40.0)
Limited SSc	22 (33.0)[k]	24 (36.0)	21 (31.0)	7 (10.4)	32 (47.8)	28 (41.8)[l]	14 (21.0)[m]	29 (45.0)	22 (34.0)
Controls	17 (11.8)	71 (49.6)	55 (38.6)	7 (5.0)	53 (37.6)	81 (57.4)	10 (7.2)	65 (46.8)	64 (46.0)

[a] $P = 0.0005$.
[b] $P = 0.04$.
[c] $P = 0.002$ (total SSc *vs* controls).
[d] $P = 0.005$.
[e] $P = 0.03$.
[f] $P = 0.003$ (total SSc; RNAP+ *vs* RNAP−).
[g] $P = 0.04$ (total SSc; U1 RNP+ *vs* U1 RNP−).
[h] $P = 0.03$.
[i] $P = 0.03$ (diffuse SSc *vs* controls).
[j] $P = 0.02$ (diffuse SSc; RNAP+ *vs* RNAP−).
[k] $P = 0.0002$.
[l] $P = 0.03$.
[m] 0.003 (limited SSc *vs* controls).

with diffuse disease as compared to controls (27.5 vs 46.8%, $P = 0.03$, OR $= 0.42$, CI 0.18–0.93).

As autoantibodies in SSc are associated with disease subgroup and prognostic factors, we analyzed the distribution of IL-10 genotypes among patients with and without disease-associated autoantibodies. The distribution of IL-10 genotypes was significantly different between Caucasian patients positive for antibodies to RNAP and U1 RNP compared to those without these autoantibodies (Table 1). At −3575 in SSc patients as a whole, the frequency of TT homozygotes was higher in subjects with antibodies to RNAP compared to those without these antibodies (60 vs 27.1%, $P = 0.005$, OR $= 4.0$, CI 1.46–11.15); this was primarily due to an increased frequency of this genotype in diffuse patients positive for the antibody (58.8 vs 19%, $P = 0.02$). The frequency of GG homozygotes at −2849 and that of CC at −2763 was higher in patients with anti-RNAP antibodies compared to those without these autoantibodies (65 vs 39.3%, $P = 0.03$, OR $= 2.8$, CI 1.03–7.94; 70 vs 34.1% $P = 0.003$, OR $= 4.5$, CI 1.5–12.9). In subjects with antibodies to U1 RNP, the frequency of the −2763 CC genotype was lower than in those without these autoantibodies (10 vs 44.5%, $P = 0.04$). IL-10 genotypes were not associated with anti-topo I or ACA (data not shown).

The distribution of IL-10 genotypes in Japanese SSc patients and controls, and in SSc patients with and without ACA, U1 RNP, and topo I autoantibodies is presented in Table 2. Interestingly, no subjects had the AA genotype at any loci. Examination of the patient population as a whole showed no significant difference

in genotype frequencies between patients and controls. Subgroup analysis, however, revealed that in diffuse patients at −2763, the frequency of AC heterozygotes was higher (21 vs 8.6%, $P = 0.04$, OR $= 2.8$, CI 1.02–7.79) and that of CC homozygotes lower (79 vs 91.4%, $P = 0.04$, OR $= 0.35$, CI 0.13–0.97) than the respective frequencies in the control population.

In the total patient population, the frequency of AT heterozygotes at −3575 was higher (20.8 vs 6.8%, $P = 0.03$, OR $= 3.6$, CI 1.04–12.58) and that of TT homozygotes lower (79.2 vs 93.2%, $P = 0.03$, OR $= 0.27$, CI 0.08–0.97) in patients with ACA than in those without these autoantibodies. Patients with limited SSc accounted for the increased frequency of the AT genotype in ACA-positive subjects (20.8 vs 3.2%, $P = 0.02$). The AT heterozygotes at this locus were also more prevalent in the diffuse SSc patients with anti-U1 RNP antibodies than in those lacking these autoantibodies (75 vs 5.4%, $P = 0.004$). At −2763, the frequency of AC heterozygotes was higher (24.1 vs 6.1%) and that of CC homozygotes lower (75.9 vs 93.9%) in patients with antibodies to topo I than in those without these autoantibodies ($P = 0.005$). Patients with limited SSc were primarily responsible for the increased frequency of AC heterozygotes in anti-topo I-positive subjects (22.6 vs 5.5%, $P = 0.03$). IL-10 genotypes were not associated with antibodies to RNAP. Other genotype frequencies were similar between total patients or subgroups and controls (data not shown).

In Caucasians, homozygosity for the A allele at −3575 and −2763 was associated with over three-fold higher risk of developing SSc. The protective effects of IL-10 against fibrosis, a hallmark feature of scleroderma, could

Table 2 Distribution of IL-10 genotypes and prevalence of autoantibodies in Japanese subjects[a]

Subjects, N (%)	−3575 genotype		−2849 genotype		−2763 genotype	
	AT	TT	AG	GG	AC	CC
Total SSc	12 (9.5)	115 (90.5)	2 (1.6)	122 (98.4)	18 (14.5)	106 (85.5)
ACA+	5 (20.8)[b]	19 (79.2)	2 (8.7)	21 (91.3)	1 (4.0)	24 (96)
ACA−	7 (6.8)	96 (93.2)	0	101 (100)	17 (17.2)	82 (82.8)
Topo I+	7 (11.9)	52 (88.1)	0	59 (100)	14 (24.1)[c]	44 (75.9)
Topo I−	5 (7.4)	63 (92.6)	2 (3.1)	63 (96.9)	4 (6.1)	62 (93.9)
Diffuse SSc	5 (12.2)	36 (87.8)	0	40 (100)	8 (21)[d]	30 (79.0)
U1 RNP+	3 (75)[e]	1 (25)	0	4 (100)	2 (66.7)	1 (33.3)
U1 RNP−	2 (5.4)	35 (94.6)	0	36 (100)	6 (17.1)	29 (82.9)
Limited SSc	7 (8.1)	79 (91.2)	2 (2.3)	82 (97.6)	10 (11.6)	76 (88.4)
ACA+	5 (20.8)[f]	19 (79.2)	2 (8.7)	21 (91.3)	1 (4.0)	24 (96)
ACA−	2 (3.2)	60 (96.8)	0	61 (100)	9 (14.8)	52 (85.2)
Topo I+	2 (6.7)	28 (93.3)	0	29 (100)	7 (22.6)[g]	24 (77.4)
Topo I−	5 (8.1)	51 (91.1)	2 (3.6)	53 (96.4)	3 (5.5)	52 (94.5)
Controls	6 (4.4)	131 (95.6)	3 (2.4)	124 (97.6)	10 (8.6)	106 (91.4)

[a]No subjects had the AA genotype.
[b]$P = 0.03$ (total SSc; ACA+ vs ACA−).
[c]$P = 0.005$ (total SSc; topo I+ vs topo I−).
[d]$P = 0.04$ (diffuse SSc vs controls).
[e]$P = 0.004$ (diffuse SSc; U1 RNP+ vs U1 RNP−).
[f]$P = 0.02$ (limited SSc; ACA+ vs ACA−).
[g]$P = 0.03$ (limited SSc topo I+ vs topo I−).

contribute to the immunological mechanisms underlying these associations. IL-10 reduces tumor necrosis factor-α (TNF-α)-induced proliferation of fibroblasts and decreases production of type 1 collagen and fibronectin by fibroblasts.[2,3] As mentioned before, the quantitative expression of IL-10 is highly heritable, and IL-10 genotypes contribute to this phenomenon. Indeed, the risk-conferring genotypes in Caucasians in this study— AA (−3575) and AA (−2763)—are strongly associated with low production of this cytokine.[6]

In the Japanese subjects, there were no AA homozygotes and the only genotype at −2763 carrying the A allele, the AC heterozygote, was associated with over two-fold increased risk of diffuse SSc. Thus, in these A allele carrying subjects, the IL-10 levels may be too low to afford protection from fibrosis, resulting in a higher risk of developing scleroderma. Homozygosity for the G allele at −2849 was marginally associated with protection from the disease in Caucasians, which is in line with the reported over-representation (although not statistically significant) of this allele in IL-10 high producers.[6]

Since fibrosis is more extensive in patients with diffuse SSc than limited SSc, in our subgroup analyses we had expected the diffuse patients to account for most of the variation observed in the total patient population. This appears to be the case for the Japanese subjects, where the frequency of AC heterozygotes was higher in diffuse, but not in limited, patients than that in the controls. In Caucasians, however, patients from both categories contributed to the overall variation at −2763, while those with the limited form of the disease were the primary contributors to the differences at −3575 and −2849 loci. Clearly, other factors, in addition to IL-10 genotypes, contribute to the clinical phenotype of SSc patients. The results presented here are at variance with those reported by Crilly et al,[18] who found no association between IL-10 genotypes and SSc as a whole, but reported a decreased frequency of a genotype associated with high IL-10 production in patients with the diffuse form of the disease. The two studies, however, are not comparable: Crilly et al studied the IL-10 sites in the proximal region of the promoter, whereas we examined distal SNPs. Other differences include the study population—homogeneous Scottish Caucasians in the study by Crilly et al vs heterogeneous North American Caucasians and homogeneous Japanese in the present study.

In addition to being risk factors for the development of the disease, particular genotypes in both Caucasian and Japanese populations contributed to disease-related autoantibodies associated with different clinical phenotypes of scleroderma. In Caucasian subjects, homozygosity for the T, G, and C alleles at −3575, −2849, −2763, respectively, was associated with the presence of anti-RNAP antibodies. At −3575, patients with the diffuse form of the disease contributed to this association; at the other two loci, no disease subtype-associated differences were found. In the Japanese patients, heterozygosity at −3575 was associated with the prevalence of ACA, while that at −2763 was associated with the presence of anti-topo I antibodies. Patients with the limited form of the disease contributed to the variation at both loci.

Since this is the first study of this type for scleroderma, these results cannot be compared directly with other studies. Involvement of IL-10 genotypes in humoral immunity to autoantigens is in accord with the enhan-

cing effect of this cytokine on B-cell survival. However, no consistent pattern of autoreactivity, explainable by the known associations between particular genotypes and the cytokine levels, emerges. It might be relevant to point out that the autoantibodies examined here are strongly associated with particular HLA alleles, and for ACA, a stronger association has been found with certain promoter region determinants of the TNF-α gene.[14,19] Perhaps simultaneous examination of HLA, TNF-α and IL-10 loci in a large study population would shed further light on humoral autoreactivity in scleroderma.

The reason for the observed ethnic differences in genetic association is not clear. Genetic heterogeneity might in part explain the ethnic disparities observed here.[20] Differences in allele frequencies and linkage disequilibrium among populations originating from different continents may also contribute to ethnically restricted associations.[21] Additionally, the low frequency of A alleles in the Japanese subjects may have reduced the statistical power to detect an association between IL-10 genotypes and disease risk in this ethnic group. In addition to differences in allele frequencies between the groups, it is likely that multiple genes interact in an epistatic manner to cause SSc, and differences in gene frequencies at these loci result in differences in relative risk to develop the disease in different ethnic groups. Evidence for such epistatic interactions in SSc has been presented elsewhere.[15]

In addition to the possible influence of IL-10 genes (via their gene products) on SSc pathogenesis discussed above, the observed associations could also be explained by linkage disequilibrium between the IL-10 alleles and those of another, as yet unidentified, locus for SSc. Such case–control studies cannot distinguish between the two possibilities. To our knowledge, this is the first report of an association between IL-10 genotypes and SSc-related autoimmune responses. Although some of the IL-10 genotype associations with SSc and SSc-related autoimmune responses reported here are highly significant and can, at least partially, be explained by the known immunological properties of IL-10, they must be followed by confirmation in an independent study population to be of wider significance.

Acknowledgements

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