Gene Polymorphisms of Tissue Plasminogen Activator and Plasminogen Activator Inhibitor-1 in Patients with Antiphospholipid Antibodies

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ABSTRACT. ***Objective.*** Impaired fibrinolytical outcomes may be one of the pathogenic factors for thrombotic events in patients with antiphospholipid antibodies (aPL). We investigated the consequences of the gene polymorphisms of tissue plasminogen activator (tPA) and plasminogen activator inhibitor-1 (PAI-1) in patients positive for aPL.

Methods. Seventy-seven Japanese and 82 British patients with aPL were examined for Alu-repeat insertion (I)/deletion (D) polymorphism of the tPA gene by polymerase chain reaction (PCR), and 4G/5G polymorphism in the PAI-1 promoter gene by site-directed mutagenesis-PCR and restriction fragment length polymorphism analysis. Correlations between these polymorphisms and clinical symptoms of antiphospholipid syndrome (APS) (arterial thrombosis, venous thrombosis, miscarriage) were analyzed.

Results. Significant differences in the allele frequencies of these genes did not exist between patients and controls. There was no significant correlation between these gene polymorphisms and clinical symptoms of APS in patients with aPL.

Conclusion. Polymorphisms of the tPA or PAI-1 genes probably do not significantly influence the risk of arterial thrombosis, venous thrombosis, or pregnancy morbidity in patients with aPL. (J Rheumatol 2002;29:1192–7)

Key Indexing Terms:
FIBRINOLYSIS GENOME PATHOGENESIS
ANTIPHOSPHOLIPID ANTIBODY SYNDROME THROMBOSIS

Antiphospholipid antibody syndrome (APS) is one of the most frequent causes of acquired thrombophilia. Detection of antiphospholipid antibodies (aPL) is carried out mostly by β_2-glycoprotein I dependent anticardiolipin assays and lupus anticoagulant (LAC) assays. The clinical value of these assays is widely acknowledged, and the tests are included in the classification criteria of APS[1]. The major clinical features of this syndrome are recurrences of arterial thrombosis, venous thrombosis, and pregnancy loss. Other clinical manifestations include thrombocytopenia, neurological disorders, hemolytic anemia, migraine, and pulmonary hypertension. The thrombotic events tend to recur: arterial thrombosis is often followed by another arterial thrombotic event and likewise for venous thrombosis[2]. An explanation for this heterogeneity and the tendency toward recurrence has remained elusive. A well-accepted mechanism of pregnancy loss is placental insufficiency due to vascular occlusion[3], although several other mechanisms have been proposed.

Antiphospholipid antibodies are a significant factor in the pathophysiology of thrombotic events: aPL impair anticoagulants such as protein C or antithrombin III[4,5], activate platelet function[6], induce a procoagulant state by activating endothelial cells[7], or induce early atherosclerotic lesions at least in animal models[8]. However, no correlation between methods of detection of aPL and other clinical symptoms has been found, suggesting that APS is a multifactorial disorder in which some factors that may not directly relate to aPL may nevertheless influence the occurrence and nature of thrombotic events.

Dysregulation of fibrinolysis may also play a significant role in determining the clinical course of patients with aPL. Tissue plasminogen activator (tPA) and plasminogen activator inhibitor-1 (PAI-1) are 2 major regulators in thrombolysis. Plasmin is converted from plasma plasminogen by tPA, a serine protease that promotes the initial step in the fibrinolytic cascade on the surface of fibrin. On the other hand, PAI-1 is one of the most potent serine protease inhibitors that inactivate tPA. Polymorphisms of Alu-repeat insertion (I)/deletion (D) in intron h of the tPA gene, and 4G/5G in the 5'-untranslated region at -675 of the PAI-1 gene are known, as are the

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differences in productivity of these proteins between genotypes[9,10]. Whether these polymorphisms affect the occurrence of thrombosis and their relationships with arterial or venous thrombosis have been given attention[11-17]. We speculated that impairment of fibrinolysis induced by the tPA and PAI-1 gene polymorphisms may modulate the initiation or the severity of thrombotic events in APS and analyzed these genetic factors in Japanese and Caucasian patients positive for aPL.

MATERIALS AND METHODS

Patients and controls. This retrospective study comprised 77 Japanese (8 men, 69 women) and 82 Caucasian patients (10 men, 72 women), all positive for aPL. Their clinical records were carefully reviewed and the clinical symptoms such as arterial thrombosis, venous thrombosis, and pregnancy loss were characterized. Of the 77 Japanese patients, 41 were diagnosed as APS [14 primary APS, 21 secondary to systemic lupus erythematosus (SLE), and 6 APS with autoimmune disorders other than SLE]. Thirty-six subjects, albeit positive for aPL, had no clinical manifestations of APS, and had other autoimmune disorders (26 SLE, 4 rheumatoid arthritis, 4 Sjögren's syndrome, one mixed connective tissue disease, 1 polymyalgia rheumatica). Of these patients, 17 were taking warfarin, 36 were being treated with antiplatelet agents, and 48 with corticosteroids. The followup period varied from 2 to 27 years with an average of 11.2 years. Of the 82 Caucasian patients, 76 were diagnosed as APS (51 primary APS, 25 secondary to SLE). Three had SLE and 3 thrombocytopenia, all positive for aPL. Of these patients, 40 were taking warfarin, 43 were treated with antiplatelet agents, and 23 with corticosteroids. The followup period varied from 1 to 38 years with an average of 10.1 years. Diagnoses of APS and SLE were based on proposed criteria for APS[1] and American College of Rheumatology criteria for classification of SLE[18]. Clinical features of these patients are given in Table 1. Arterial thromboses were diagnosed by magnetic resonance imaging, computed tomography, or angiography. Venous thromboses were diagnosed by venography or radioisotope-labelled venography. Pulmonary embolisms were diagnosed using perfusion scintigraphy. DNA samples were obtained from all these patients. DNA from 144 healthy Japanese and 38 healthy Caucasians served as controls. Written permission was obtained from each patient to use DNA for this purpose. As the patients had antithrombotic therapy after the first thrombotic event, the difference of the treatment did not confound the results. Among each subgroup of patients, no difference was found in the length of followup.

Analysis of Alu-repeat polymorphism in the tPA gene. I/D polymorphism resulting from the presence/absence of an Alu repeat in the exon h of the tPA gene was identified by polymerase chain reaction (PCR) and electrophoresis. We utilized primers described by Yang-Feng, *et al*[19]: 5' primer; 5'-TCCGTAACAGGACAGCTCA-3' (PR-TPAOL-1; nt 25,216-25,234), 3' primer: 5'-ACCGTGGCTTCAGTCATGGA-3' (PR-TPAOL-2; nt 26,181-26,162). PCR conditions were as follows: 10 µl of reaction liquid containing 5 pmol of each primer, 1 µl of 10 × PCR buffer II, 0.8 µl of dNTP mixture (2 mM of each dNTP), 0.6 µl of $MgCl_2$ (25 mM), and 0.08 µl of ampli Taq Gold (Perkin-Elmer, Norfolk, CT, USA), was subjected to the initial denature step for 9 min at 94°C, followed by 35 cycles of denaturing at 94°C for 1 min, annealing step at 56°C for 1 min, extension step at 72°C for 2 min, and an additional extension step at 72°C for 5 min. PCR products were electrophoresed in 2% agarose gels. Nine hundred and sixty-seven bp and 655 bp bands correspond to I and D alleles, respectively.

Table 1. Clinical features of the patients. Values are the number of positive/number of patients tested (%).

	Japanese	Caucasian
Arterial thrombosis	21/77 (27.3)	41/82 (50.0)
Venous thrombosis	7/77 (9.1)	41/82 (50.0)
Pregnancy loss	10/51 (19.6)	30/72 (41.7)
Anticardiolipin	39/77 (50.6)	60/82 (73.2)
Lupus anticoagulant	56/73 (76.7)	49/82 (59.8)

Analysis of 4G/5G polymorphism in PAI-1 promoter gene. Site-directed mutagenesis PCR (SDM-PCR) and subsequent restriction fragment length polymorphism (RFLP) analysis, using DraIII (New England Biolabs, Beverly, MA, USA) were done to analyze the 4G/5G polymorphism of the PAI-1 promoter gene. DraIII restriction enzyme site was introduced into amplification products of the 4G allele by SDM-PCR. The upstream primer, described by Kimura, *et al*[20], 5'-TCCAACCTCAGCCAGACAAG-3' (PAI-1 pr1) and the newly designed downstream primer 5'-TGATACACGGCTGACTCACC-3' (underlining indicates the mutation introduced to form the restriction site for DraIII) were utilized. PCR was done in the same fashion except for the annealing temperature (65°C) and each reaction time of denaturing, annealing, and extension steps (30 s). Five microliter of SDM-PCR products were digested overnight with 10 units of Dra-III at 37°C and resolved on a 4% agarose gel. DraIII digests the amplified products from the 4G alleles into 2 fragments of 71 and 19 bp. Products amplified from genes with the 5G allele remain undigested (Figure 1). The accuracy of SDM-PCR and RFLP system for detection of the PAI-1 4G/5G gene polymorphism was confirmed by sequence analysis in several randomly selected samples.

Determination of aPL. Anticardiolipin enzyme immunoassays and LAC assays were done in these patients. IgG/M aCL were measured by standard aCL ELISA[21]. Existence of LAC was determined, according to guidelines recommended by the Subcommittee on Lupus Anticoagulant/Phospholipid-dependent Antibodies[22], by prolonged activated partial thromboplastin time, dilute Russell's viper venom time or kaolin clotting time, and their correction by phospholipid or platelets.

Statistical analysis. Correlations between the polymorphisms of tPA and PAI-1 genes and clinical symptoms (arterial thrombosis, venous thrombosis, pregnancy loss) were analyzed. Contingency table analyses were done using Fisher's exact test. Both univariate and multivariate analysis were done. P values ≤ 0.05 were considered to have statistical significance.

RESULTS

Genotypes and allele frequencies of I/D polymorphism of tPA and 4G/5G polymorphism of PAI-1 genes of both Japanese and Caucasian healthy volunteers are similar to those documented[14,20,23]. As shown in Table 2, these data are consistent with the distribution predicted by the Hardy-Weinberg equilibrium. Significant differences in both genotypes and allele frequencies of both polymorphisms of tPA and PAI-1 genes did not exist between patients and controls, or between Caucasian and Japanese.

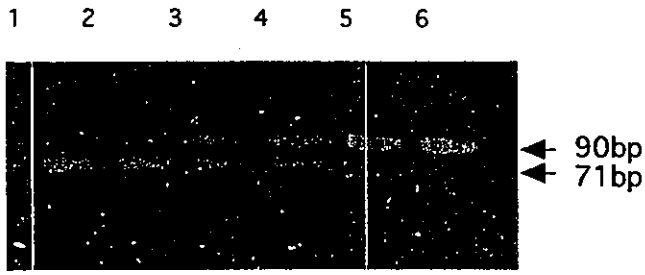

Figure 1. Analysis of the plasminogen activator inhibitor-1 (PAI-1) gene. Site-directed mutagenesis PCR and subsequent RFLP analysis were used to analyze 4G/5G polymorphism in the promoter region of PAI-1 gene. The Dra III restriction site was introduced into 4G alleles (71+19bp), while the 5G alleles remained undigested (90 bp). Lane 1 and 2: homozygous for 4G allele; lane 3 and 4: 4G/5G; lane 5 and 6: homozygous for the 5G allele.

Table 2. Prevalence of I/D polymorphism of tPA gene and 4G/5G polymorphism of PAI-1 gene. Values are numbers of genotypes or alleles (%).

Genotype	tPA II	tPA DI	tPA DD	PAI-1 4G/4G	PAI-1 4G/5G	PAI-1 5G/5G
Japanese						
aPL+ patients	22 (28.6)	35 (45.5)	20 (26.0)	23 (29.9)	45 (59.7)	8 (10.4)
Healthy controls	43 (30.0)	66 (45.8)	35 (24.3)	38 (26.4)	73 (50.7)	33 (22.9)
Caucasian						
aPL+ patients	30 (35.7)	34 (40.5)	18 (21.4)	25 (30.5)	38 (46.3)	19 (23.2)
Healthy controls	9 (23.7)	21 (55.3)	8 (21.1)	10 (26.3)	22 (57.9)	6 (15.8)

Allele	I Allele	D Allele	4G Allele	5G Allele
Japanese				
aPL+ patients	79 (51.3)	75 (48.7)	92 (59.7)	62 (40.3)
Healthy controls	152 (52.8)	136 (47.2)	149 (51.7)	139 (48.3)
Caucasian				
aPL+ patients	90 (57.3)	70 (42.7)	88 (53.7)	76 (46.3)
Healthy controls	39 (51.3)	37 (21.1)	42 (55.3)	34 (44.7)

aPL: antiphospholipid antibodies; tPA: tissue plasminogen activator, PAI-1: plasminogen activator inhibitor-1.

In monovariate analysis, I allele of tPA, when compared with D allele, had a weak correlation with a history of arterial thrombosis (p = 0.029) in Japanese patients, while in Caucasians, D allele of tPA, compared with I allele, had a weak correlation with a history of venous thrombosis (p = 0.018). Statistical significance was lost when multivariate analyses were done (Table 3A). There was no significant correlation between the gene polymorphism of PAI-1 and clinical symptoms (Table 3B). No allele investigated in this study was commoner in patients with multiple symptoms than in the others (Table 3). We also investigated the association between APS symptoms and both of I/I genotype of tPA (compared with non-I/I genotypes) and 4G/4G genotype (compared with non-4G/4G genotypes), finding no significant correlations (Table 4).

DISCUSSION

The consequences of tPA or PAI-I polymorphisms have been discussed. In 1992, Ludwig, *et al*[23] found an Alu-repeat I/D polymorphism in intron h of the tPA gene. Basal tPA release rates were unaffected by this polymorphism when human umbilical vein endothelial cells were used[24], but *in vivo*,

Table 3A. Correlation between clinical features in patients with antiphospholipid antibodies and alleles of tissue plasminogen activator. Odds ratio as the relative risk of I allele for having each manifestation.

		Allele Count I Allele	Allele Count D Allele	Odds Ratio	95% CI	p Value Univariate	p Value Multivariate
Japanese							
Arterial thrombosis	(+)	28	14				
	(–)	51	61	2.39	1.14–5.02	0.029	0.876
Venous thrombosis	(+)	5	9				
	(–)	74	66	0.50	0.16–1.55	0.269	
Pregnancy loss	(+)	13	7				
	(–)	42	40	1.77	0.64–4.88	0.323	
Multiple symptoms	(+)	6	2				
	(–)	88	68	2.32	0.45–11.85	0.468	
Caucasian							
Arterial thrombosis	(+)	47	35				
	(–)	47	35	1.00	0.54–1.86	> 0.9999	
Venous thrombosis	(+)	39	43				
	(–)	55	27	0.45	0.24–0.84	0.018	0.053
Pregnancy loss	(+)	33	27				
	(–)	50	34	0.83	0.43–1.62	0.616	
Multiple symptoms	(+)	21	25				
	(–)	72	45	0.53	0.26–1.05	0.793	

Table 3B Correlation between clinical features in patients with antiphospholipid antibodies and alleles of plasminogen activator inhibitor-1. Odds ratio as the relative risk of I allele for having each manifestation.

		Allele Count 4G Allele	5G Allele	Odds Ratio	95% CI	p Value Univariate
Japanese						
Arterial thrombosis	(+)	20	22			
	(−)	72	40	0.51	0.25–1.03	0.067
Venous thrombosis	(+)	9	5			
	(−)	83	57	1.24	0.39–3.88	0.783
Pregnancy loss	(+)	13	7			
	(−)	44	38	1.60	0.58–4.43	0.454
Multiple symptoms	(+)	6	2			
	(−)	86	60	2.09	0.41–10.73	0.476
Caucasians						
Arterial thrombosis	(+)	42	40			
	(−)	46	36	0.82	0.44–1.52	0.639
Venous thrombosis	(+)	42	40			
	(−)	46	36	0.82	0.44–1.52	0.639
Pregnancy loss	(+)	35	25			
	(−)	43	41	1.34	0.68–2.60	0.742
Multiple symptoms	(+)	26	20			
	(−)	62	56	1.17	0.59–2.33	0.728

Table 4. Associations of I/I genotype of tPA or 4G/4G genotype of PAI-1 gene and APS symptoms. Values are number of patients positive/number of patients tested. p Values of I/I genotype vs non-I/I genotypes and 4G/4G genotype vs non-4G/4G genotypes were calculated.

	Genotypes of tPA I/I	Non-I/I	p	Genotypes of PAI-1 4G/4G	Non-4G/4G	p
Japanese						
Arterial thrombosis	10/22	11/54	0.122	3/23	18/53	0.174
Venous thrombosis	1/22	6/54	0.667	2/23	5/53	> 0.999
Pregnancy loss	4/14	6/37	0.462	5/14	5/37	0.261
Multiple symptoms	2/22	2/54	0.579	2/23	2/53	0.585
Caucasians						
Arterial thrombosis	15/30	9/52	> 0.999	12/25	29/57	> 0.999
Venous thrombosis	9/30	31/52	0.150	13/25	28/57	> 0.999
Pregnancy loss	10/26	20/46	0.824	10/22	20/50	0.817
Multiple symptoms	4/30	19/52	0.129	8/25	15/57	0.800

release rates were markedly higher in subjects homozygous for the I allele[9]. In a study done in The Netherlands, the I allele was significantly more frequent than the D allele in patients with myocardial infarction[11]. However, subsequent studies in other populations found no such association[12,13].

In 1993, 4G/5G polymorphism located in the 5'-untranslated region at position -675 of the PAI-1 gene was described by Dawson, *et al*[10], and an *in vitro* study using HepG2 cells indicated that the 4G allele is associated with enhanced gene expression under stimulation. In addition, there are reports showing that the 4G allele corresponds to significantly higher PAI-1 levels than does the 5G allele[15,25-27]. In studies done in Sweden, the 4G allele was reported to be a risk for myocardial infarction[25] and deep vein thrombosis[16]. In contrast, other studies found that this polymorphism was not a risk factor for thrombosis[26-28].

A possible relationship between impaired fibrinolysis and APS has to be considered. These polymorphisms may be used as a discriminator value to stratify patient populations with respect to incremental function of these proteins. In patients with aPL, some studies noted impairments in the fibrinolytic system, which is vital for preventing clot formation and occlusion in vessels. Francis, *et al*[29] reported that, in some patients with LAC and thrombosis, increments in tPA activity after venous occlusion were not evident. Jurado, *et al*[30] reported higher PAI-1 levels before and after venous occlusion in patients with autoimmune diseases. Decreased tPA release and higher mean PAI-1 levels were investigated[31,32], although in

the former report there was no correlation with high level of aPL. Reduced fibrinolytic response to venous occlusion was also noted in patients with aPL who did not have SLE[33].

We speculated that the reported perturbation in fibrinolysis by tPA and PAI-1 gene polymorphisms may alter the risks of thrombosis in APS patients more significantly than in patients with other thrombotic disorders. However, our current study, revealed neither the I allele of tPA nor the 4G allele of PAI-1 to be significant risk factors for thrombosis or pregnancy loss in patients positive for aPL. While preparing our study, the positive correlation between PAI-1 polymorphism and thrombosis in APS was reported in a Catalan population[34]. In Japanese and in British Caucasians, however, we found no correlation between these gene polymorphisms and symptoms of APS. Polymorphism of the tPA and the PAI-1 gene does not seem to significantly influence the risk of thrombosis or other symptoms in patients with APS.

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Anti-β_2-glycoprotein I antibodies in children with atopic dermatitis

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Keywords: allergy, antigen, antiphospholipid antibody, autoantibody, autoimmunity, epitope

Abstract

β_2-Glycoprotein I (β_2GPI) appears to be the major antigen for antiphospholipid antibodies (aPL) in patients with antiphospholipid syndrome (APS). In early infancy, virtually all children initiate transient immune response to non-pathogenic nutritional antigens, which fails to terminate in children with atopic diseases. To examine the possibility that a prolonged immune response to β_2GPI could also spread to the human protein, antibodies against human β_2GPI (anti-β_2GPI) were determined in 93 randomly selected children with different allergic diseases. A high frequency (42%) of IgG anti-β_2GPI was found in children with atopic dermatitis (AD), but not in those with other allergic diseases. Anti-β_2GPI in children with AD were exclusively of the IgG1 subclass and bound to bovine β_2GPI as well, but not to either β_2GPI combined with the phospholipid cardiolipin. The epitopes were identified in domain V of β_2GPI and the antibody binding was abolished upon the specific proteolytic cleavage of the phospholipid-binding C-terminal loop in domain V of β_2GPI. These results indicated that the epitopes for anti-β_2GPI in children with AD most likely resided in close vicinity of the phospholipid-binding site of β_2GPI. The epitopic difference from anti-β_2GPI in APS may explain presumed non-thrombogenicity of anti-β_2GPI in children with AD.

Introduction

Antiphospholipid antibodies (aPL) are detected in a number of clinical disorders including autoimmune, infectious, malignant and other diseases. However, thrombosis and recurrent fetal loss associated with aPL, constituting the antiphospholipid syndrome (APS), are reported in autoimmune patients only (1). Originally thought to be directed towards negatively charged phospholipids, aPL in autoimmune patients are now known to recognize certain phospholipid-binding plasma proteins, thereby differing from aPL in other diseases, which bind directly to phospholipids (2,3). β_2-Glycoprotein I (β_2GPI) appears to be by far the commonest and best-characterized antigenic target for presumably pathogenic aPL (4). β_2GPI is a single-chain (326 amino acids) 50-kDa plasma protein composed of five homologous short consensus repeat domains (5,6). Domain V of β_2GPI differs from the other four domains by two additional cysteins, responsible for the second internal loop and the long C-terminal tail (7). Domain V also possesses a highly positively charged amino acid sequence Cys281–Cys288, which was shown to be a potent phospholipid-binding site (8). Recent X-ray analysis of β_2GPI showed that a patch consisted of 14 positively charged amino acid residues on domain V, as well as the flexible and partially hydrophobic C-terminal loop between Ser311 and Lys317, are involved in the binding to phospholipids (9). The binding of β_2GPI to phospholipids could significantly be reduced by the specific proteolytic cleavage of the C-terminal loop between Lys317 and Thr318 (10,11).

The exact epitopic sites for antibodies against β_2GPI (anti-β_2GPI) have not yet been revealed, but it seems that domains I and IV are dominant in the binding of anti-β_2GPI from APS patients (12–14). The physiological function of β_2GPI is still unclear. However, the interaction of β_2GPI and anti-β_2GPI

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apparently leads to a prothrombotic state, accelerated atherogenesis, and interferes with the clearance and processing of apoptotic cells.

Particularly during the first few months of life, intact macromolecules may pass through the epithelium of the gastrointestinal tract in amounts sufficient to effectively engage the T cell system (15). It has been shown that virtually all children initiate IgG1 production against nutritional antigens by 3 months of age, and this production typically peaks during infancy and wanes thereafter, whereas corresponding IgG4 responses continue to develop (16). This early pattern of transient T_h2-dependent antibody production against non-pathogenic nutritional antigens fails to terminate in children with atopic diseases (17,18). Since β_2GPI has been remarkably conserved during the evolution of animal species, showing >80% homology between the amino acid sequences of human, bovine and rat counterparts (19), we have considered the possibility that in children with atopic diseases an exaggerated immune response to β_2GPI from milk or meat products could lead not only to the production of antibodies against bovine β_2GPI, but also toward human β_2GPI. The immunization of experimental animals with heterologous β_2GPI was shown to induce the production of antibodies specific for the administered antigen and a subset of antibodies that cross-reacted with homologous β_2GPI (20). To our knowledge, no links between anti-β_2GPI and allergic diseases have been reported yet.

We report here a high frequency of anti-β_2GPI in children with atopic dermatitis (AD), but not in those with other allergic diseases examined. Anti-β_2GPI in children with AD were found to recognize epitopes on domain V of β_2GPI, most probably in close vicinity of the phospholipid-binding site. This epitopic difference from anti-β_2GPI in APS and the inability of AD-associated anti-β_2GPI to bind β_2GPI in combination with phospholipids may provide clues to understanding their presumed non-thrombogenicity.

Methods

Serum samples

Five groups of sera were selected from the serum banks of the Pediatrics Clinic and Department of Rheumatology (University Medical Centre, Ljubljana). The main group comprised 71 consecutive sera from children with different atopic diseases positive for at least one allergen-specific IgE antibody or having elevated total serum IgE. Among them, 45 children suffered from AD, 36 exhibiting pure-type AD, while nine also had other types of allergy associated with AD, i.e. mixed-type AD (27 boys and 18 girls, mean age 3.7 years, range 2 months to 16.8 years). The other 26 children had atopic diseases with respiratory system symptoms only (allergic rhinitis 15, asthma 3, both 8 children; 21 boys and five girls, mean age 11 years, range 1.9–18 years). The second group consisted of 22 children with a non-atopic allergic disease who had experienced a systemic anaphylactic reaction after stings of *Hymenoptera* insects (16 boys and 6 girls, mean age 11 years, range 2.1–17.8 years). The normal control group for children comprised 61 sera from apparently healthy children collected at their regular preventive visits (25 boys and 36 girls, mean age 9.4 years, range 4.9–14.2 years). As a positive control group, 26 sera from adult primary or secondary APS patients were randomly selected. The control group for adults consisted of 52 healthy blood donors (31 men and 21 women, mean age 34.0 years, range 18–65 years).

mAb

Two monoclonal β_2GPI-dependent aPL were used as calibrators to establish standard curves for the anti-β_2GPI and anticardiolipin antibody (aCL) ELISA: HCAL, a chimeric IgG mAb consisting of human κ and γ1 C regions and V regions from the mouse monoclonal aCL WBCAL-1 (21,22), and EY2C9, an IgM mAb derived from a patient with APS (23). Arbitrary units (AU) were set for the anti-β_2GPI and aCL ELISA by the concentrations of the mAb giving definite absorbance values. The activity of 1 ng/ml of HCAL was defined as 1 IgG AU (AUG) and the activity of 5 ng/ml of EY2C9 as 1 IgM AU (AUM). IgA AU (AUA) were set by the dilutions of a selected IgA anti-β_2GPI$^+$ in-house standard from a patient with APS.

Three anti-β_2GPI mAb (Cof-19, Cof-22 and Cof-23) obtained from BALB/c mice immunized with human β_2GPI (12) were applied as positive controls.

Anti-β_2GPI ELISA

β_2GPI was purified from pooled human sera by affinity column chromatography (2) and coated at 10 µg/ml in PBS (pH 7.4, 50 µl/well) on Costar High Binding EIA/RIA plates (cat. no. 3590; Costar, Cambridge, MA) for 2 h at room temperature. After one wash with 300 µl of PBS containing 0.05% Tween 20 (PBS-T), 50 µl/well of the standards and test sera diluted 1:100 in PBS-T were applied for 30 min at room temperature. Following four washes, 50 µl/well of alkaline phosphatase-conjugated goat anti-human IgG, IgM or IgA (ACSC, Westbury, NY) diluted in PBS were added for 30 min at room temperature. After four washes, 100 µl/well of *p*-nitrophenylphosphate (Sigma, St Louis, MO) dissolved at 1 mg/ml in 1 M diethanolamine buffer (pH 9.8) was applied. OD at 405 nm was first measured after 15 min and then every 3 min by a microtiter plate reader (Rainbow Spectra Thermo; Tecan, Salzburg, Austria) until an optimal fitting to the predicted OD of the standards was obtained. All sera were tested in duplicates both in wells with and without β_2GPI (sample blank values). Specific absorbance was obtained by subtracting sample blank values from raw data.

The cut-off levels for anti-β_2GPI in children were determined based on the absorbance values of control healthy children. The distribution of absorbance values was significantly skewed on the linear scale and nearly symmetrical on the log scale, the cut-off points were defined as the mean + 2SD for the log-absorbance values. Expressed in previously defined AU, the cut-off points for IgG, IgM and IgA anti-β_2GPI in children were 4.2 AUG, 2.6 AUM and 4.0 AUA respectively (24).

Fluid-phase inhibition test with β_2GPI

Except for the preparation of test samples, the assay conditions were the same as described for the anti-β_2GPI ELISA. Tested sera or Cof mAb diluted to 50% maximal binding in 1% BSA (essentially fatty acid free, cat. no. A-6003; Sigma) in PBS-T were mixed with equal volumes of β_2GPI solutions in the

same buffer. The final concentrations of β_2GPI were 100, 20, 4, 0.8, 0.16, 0.032 and 0 μg/ml. The mixtures were incubated for 45 min at room temperature, then 50 μl/well was applied to plate wells. OD was measured after 30 min of color development.

Anti-β_2GPI ELISA for IgG subclasses

Coating of β_2GPI, washing and application of serum samples were performed as in the anti-β_2GPI ELISA. Secondary mouse anti-human monospecific antibodies to IgG1 (diluted 1:250; clone HP 6069; Zymed, San Francisco, CA), IgG2 (diluted 1:500; clone MAB 1308; Chemicon, Temecula, CA), IgG3 (diluted 1:1000, clone HP-6050; Sigma) or IgG4 (diluted 1:1000, Clone HP-6025; Sigma) were applied in 50 μl/well aliquots for 1 h at room temperature. For color development, alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma) and *p*-nitrophenylphosphate were used.

Anti-BSA ELISA and fluid-phase inhibition test with BSA

Costar High Binding microtiter plates were coated with 50 μl/well of BSA dissolved at 20 μg/ml in PBS for 2 h at room temperature. All further steps were as described for the anti-β_2GPI ELISA. OD was measured after 45 min of incubation at room temperature. The cut-off point was defined as for the anti-β_2GPI ELISA.

For the inhibition test, β_2GPI and BSA were coated on the same plate as described for the anti-β_2GPI and anti-BSA ELISA respectively. Inhibition mixtures were prepared in duplicates, one containing tested serum and BSA at concentrations up to 1 mg/ml in PBS-T and the other serum diluted in PBS-T only. Following incubation for 45 min at room temperature, the mixtures were applied to wells with β_2GPI, BSA or without an antigen. All further steps were as in the anti-β_2GPI ELISA.

ELISA and fluid-phase inhibition test with bovine β_2GPI

The purification of bovine β_2GPI, ELISA and inhibition test were performed as described for human β_2GPI, which was also used as a control protein. Inhibition mixtures containing either bovine or human β_2GPI in fluid phase were applied to parallel duplicate wells coated with either protein so that all four possible antigen combinations were tested on the same plate. The final concentrations of β_2GPI in mixtures were 200, 100, 20, 4, 0.8, 0.16 and 0 μg/ml.

Standard aCL ELISA

Costar Medium Binding EIA/RIA plates (cat. no. 3591) were coated with cardiolipin as in the standard aCL ELISA. After incubation with 120 μl/well of 10% FBS (Sigma) in PBS for 1 h at room temperature, the plates were washed once with 300 μl/well of PBS. Then 100 μl/well of standards and serum samples diluted 1:100 in PBS containing 10% FBS were applied for 2.5 h at room temperature. Plates were washed 4 times with PBS and 100 μl/well of alkaline phosphatase-conjugated rabbit anti-human IgG (ACSC) were added. Following 1-h incubation at room temperature, plates were washed 4 times and 100 μl/well of substrate were applied. OD was measured as described for the anti-β_2GPI ELISA.

Modified aCL ELISA

Sumilon S microtiter plates (Sumitomo Bakelite, Tokyo, Japan) were coated with 40 μl/well of 50 μg/ml cardiolipin (Sigma) in ethanol by evaporation at 4°C overnight. After blocking with 200 μl/well of 1% BSA in PBS (1% BSA/PBS) for 1 h at room temperature, the plates were washed 3times with 300 μl/well of PBS-T. Then 50 μl/well of standards and serum samples diluted 1:100 in 1% BSA/PBS containing 10 μg/ml of β_2GPI were applied for 2 h at room temperature. Plates were washed 3 times and 50 μl/well of alkaline phosphatase-conjugated mouse anti-human IgG (Southern Biotechnology, Birmingham, AL) were added for 1 h at room temperature. After washing, the substrate was added and OD was measured as described for the anti-β_2GPI ELISA.

The cut-off values in the aCL ELISA, determined by the same method as in the anti-β_2GPI ELISA, were 13.9 AUG and 5.6 AUG for the standard and modified method respectively; 13.9 AUG corresponded to 7.6 GPL international standard IgG aCL units (25).

ELISA and inhibition tests with domain-deleted mutants (DM) of human β_2GPI

Recombinant DM of β_2GPI, lacking one or more domains, as well as whole molecule β_2GPI (WM) were produced by the baculovirus/Sf 9 insect cell vector expression system (12,26). The ELISA with different DM (DM II-V, DM III-V, DM IV-V and DM V lacking domains at the N-terminal region or DM I-III and DM I-IV lacking domains at the C-terminal region) as antigens was performed as outlined for the anti-β_2GPI ELISA. Cof mAb and rabbit polyclonal anti-β_2GPI served as positive controls.

Inhibition of binding to solid-phase β_2GPI was tested with recombinant domain V of β_2GPI (DM V) in fluid phase at its final concentrations of 50, 20, 4, 0.8, 0.16 and 0 μg/ml.

ELISA with proteolytically cleaved β_2GPI

Cleaved β_2GPI was prepared by treatment with human plasmin as described (27). The ELISA was performed on Costar High Binding plates using intact serum purified β_2GPI on the same plate for comparison.

Statistical analysis

Statistical tests were performed using subroutines from the statistical analysis package by MS Excel 6.0 for Windows. Chi-square test, *t*-test assuming equal or unequal variances and linear regression analysis were used when appropriate. Differences were considered significant whenever $P < 0.05$.

Results

Prevalence and clinical association of anti-β_2GPI in atopic children

Anti-β_2GPI of IgG isotype were detected in 20 of 71 (28%) atopic children. Interestingly, 19 of 20 IgG anti-β_2GPI$^+$ children (95%) were diagnosed with AD and the one not suffering from AD had an IgG anti-β_2GPI level of only 5.8 AUG above the cut-off. Considering the group of 45 children with AD, 19 (42%, 17 pure and two mixed type) had positive IgG anti-β_2GPI, while 26 children (58%, 19 pure and seven mixed type) were anti-β_2GPI$^-$. The difference in anti-β_2GPI positivity between pure

and mixed type AD was not significant. A statistically significant difference was found by comparing the age of AD children positive for IgG anti-β_2GPI (mean age 1.8 ± 0.8 years, range 3 months to 3.1 years) and those negative for anti-β_2GPI (5.1 ± 4.4 years, range 2 months to 16.1 years) ($P = 0.0003$). There was no difference in either the frequency or mean positive IgG anti-β_2GPI values between the children with AD and adult APS patients (Fig. 1).

Among children with increased IgG anti-β_2GPI, one also had increased IgM, two IgA, and one child both IgM and IgA anti-β_2GPI. Children negative for IgG anti-β_2GPI were negative for the other two isotypes as well. All 22 children with systemic anaphylaxis after an insect bite were negative for all three isotypes of anti-β_2GPI.

Inhibition of anti-β_2GPI with β_2GPI in fluid phase

The binding of IgG anti-β_2GPI from the children with AD to β_2GPI on microtiter plates was inhibited in a dose-dependent manner by β_2GPI in solution. Fifty percent inhibition was achieved by β_2GPI concentrations between 15 and 30 μg/ml (0.3–0.6 μM). These concentrations were ~100-fold higher than the IC_{50} of the Cof mAb (0.1–0.35 μg/ml). Anti-β_2GPI from the APS patients and HCAL mAb showed only 15–35% inhibition at 100 μg/ml (2 μM) of β_2GPI. Representative inhibition profiles are presented in Fig. 2.

Subclass specificity of IgG anti-β_2GPI in atopic children and APS patients

IgG1 was the only subclass detected in all eight randomly chosen IgG anti-β_2GPI$^+$ sera from the children with AD, whereas IgG2 was the predominant subclass present in all eight randomly selected sera from the APS patients. Additionally, low levels of IgG1 were detected in seven, IgG3 in one and IgG4 in two of the eight sera from the APS patients.

Binding to BSA in and cross-reactivity of anti-BSA with β_2GPI

Because of the high background binding exhibited by many sera of the atopic children in preliminary experiments using BSA as the blocking agent, we presumed that some sera may also contain anti-BSA. In fact, increased levels of IgG anti-BSA were found in 24 of 71 sera (34%) from atopic children. The frequency of anti-BSA was significantly higher in the children with AD (19 of 45 = 42%) than in the other atopic children (five of 26 = 19%, $P = 0.038$). No correlation between the absorbance values for anti-β_2GPI and anti-BSA in the children with atopic diseases was observed. There was also no association between the presence of anti-β_2GPI and anti-BSA in the children with AD.

Anti-BSA in 13 selected atopic children were inhibited in a dose-dependent manner by fluid-phase BSA (IC_{50} = 0.2–20 μg/ml BSA). BSA had no influence on the detection of anti-β_2GPI, thus excluding cross-reactivity of anti-BSA with β_2GPI.

Binding of anti-β_2GPI from atopic children to bovine β_2GPI

All 19 sera from the AD children containing antibodies toward human β_2GPI bound efficiently to bovine β_2GPI, suggesting a cross-reactivity. Five of 16 randomly selected sera among atopic children negative against human β_2GPI expressed a substantial binding to bovine β_2GPI. HCAL and EY2C9 mAb bound both antigens similarly to anti-β_2GPI from the children with AD. In addition, anti-β_2GPI from the APS patients bound efficiently both antigens, although some sera expressed a higher binding to human than to bovine β_2GPI (Fig. 3A).

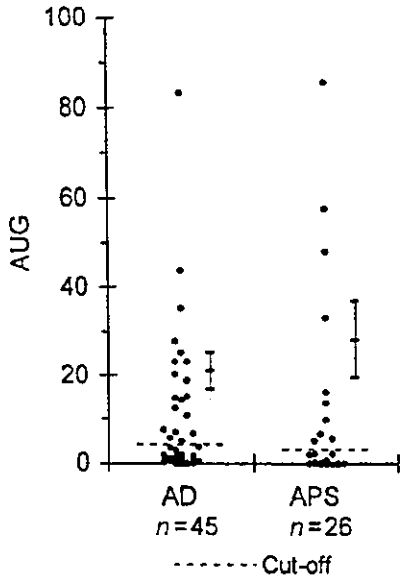

Fig. 1. Comparison of IgG anti-β_2GPI levels in children with AD and adult APS patients. No statistically significant difference in either frequency or levels of positive IgG anti-β_2GPI was found between the two groups (AD: 19/45 = 42% positive, mean 21.2 ± 18.3 AUG, range 5.5–5.5 AUG; APS: 10/26 = 38% positive, mean 28.2 ± 27.5 AUG, range 5.1–85.8 AUG). Results of IgG anti-β_2GPI ELISA are expressed in AUG defined in Methods. The cut-off values for AD and APS were 4.2 and 2.9 AUG respectively. Error bars indicate mean ± SEM of positive values.

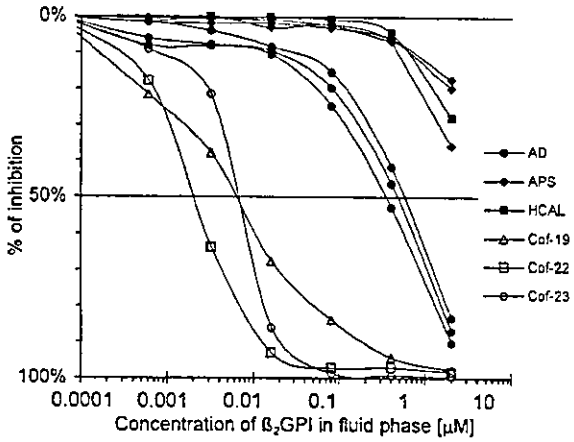

Fig. 2. Inhibition of binding to β_2GPI adsorbed on microtiter plates with β_2GPI in the fluid phase. Three representative inhibition profiles among IgG anti-β_2GPI$^+$ sera from children with AD and APS are presented. HCAL and Cof mAb were used as controls. Results are expressed as percentage of inhibition ($1 - OD_{inhibited}/OD_{non\text{-}inhibited}$).

Human and bovine β_2GPI had similar inhibitory effects on the binding to solid-phase human β_2GPI by the sera from the children with AD. However, the binding to solid-phase bovine β_2GPI was less efficiently inhibited by human than bovine β_2GPI, indicating a population of anti-β_2GPI specific for bovine β_2GPI (Fig. 3B).

Binding of IgG anti-β_2GPI from atopic children in aCL ELISA

Among 19 anti-β_2GPI$^+$ sera from the children with AD, positive results were obtained in only three and two sera by the standard and modified aCL ELISA, respectively. Among anti-β_2GPI$^-$ atopic children, three sera expressed positive values in the standard aCL ELISA and one serum in the modified aCL ELISA. No statistically significant difference in aCL positivity was observed between anti-β_2GPI$^+$ and anti-β_2GPI$^-$ sera.

Binding to DM of β_2GPI

Anti-β_2GPI from the children with AD recognized all DM containing domain V and showed no binding to DM without this domain (Fig. 4). Further, all tested anti-β_2GPI$^+$ sera from the children with AD bound to solid-phase recombinant domain V. There was also a positive correlation between the absorbance values for binding to complete β_2GPI and domain V (r = 0.85, P < 0.0001) (Fig. 5A). The binding of anti-β_2GPI from the children with AD to solid-phase β_2GPI was efficiently and in a dose-dependent manner inhibited by domain V in the fluid phase (IC_{50} = 6 to 9 µg/ml = 0.4 to 0.7 µM) (Fig. 5B). The molar concentrations of domain V and complete β_2GPI giving 50% inhibition were comparable.

Anti-β_2GPI from the APS patients bound predominantly to DM I-IV and to a lower extent to DM I-III, which was similar to the binding profiles of HCAL and EY2C9 (data not shown).

Binding to proteolytically cleaved β_2GPI

The cleavage of β_2GPI practically abolished the binding of anti-β_2GPI from all 17 tested positive children with AD (mean OD 0.386 ± 0.183, range 0.139–0.798 for native purified β_2GPI and 0.014 ± 0.018, range 0–0.060 for cleaved β_2GPI). Cof mAb, used as controls, bound to both forms of β_2GPI efficiently.

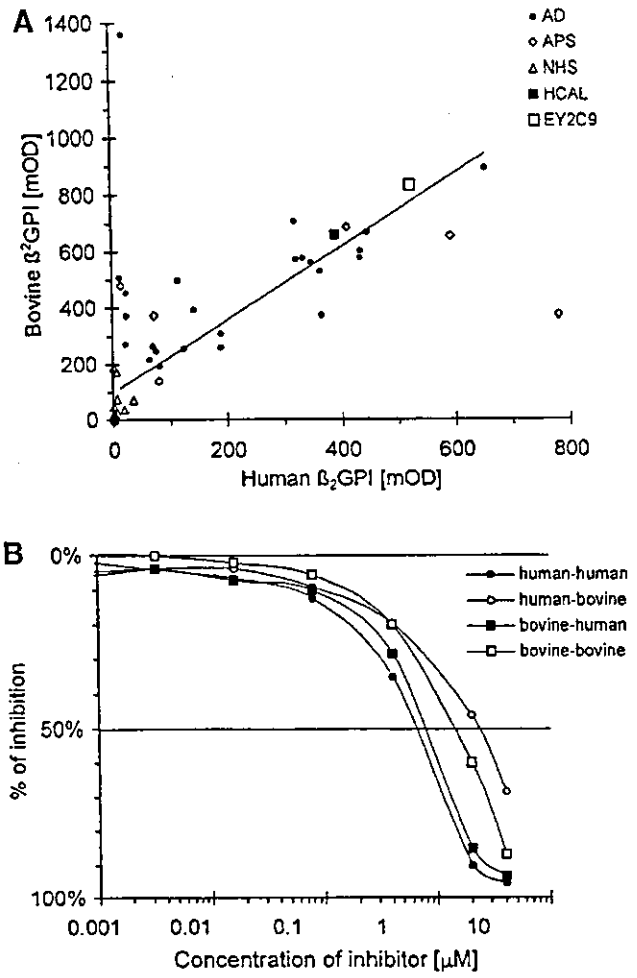

Fig. 3. (A) Binding of antibodies against human β_2GPI to bovine β_2GPI. In children with AD, the correlation between the absorbance values for human and bovine β_2GPI was statistically significant (r = 0.92, P < 0.001). Sera from patients with APS, normal human sera (NHS) and HCAL and EY2C9 mAb are shown for comparison. Results are presented as mOD (10^{-3} × OD) at 405 nm values. (B) Inhibition of binding to human or bovine GPI adsorbed on microtiter plate by either human or bovine β_2GPI in the fluid phase. Representative serum inhibition profiles from a child with AD are shown as percentage of inhibition (1 − $OD_{inhibited}/OD_{non\text{-}inhibited}$). Symbols indicate different combinations of fluid- and solid-phase antigen (first fluid- and second solid-phase β_2GPI).

Discussion

The β_2GPI requirement for the binding of aPL from patients with autoimmune disorders associated with APS is one of the features that distinguish them from aPL occurring in non-autoimmune diseases (2,3). Antibodies reacting with β_2GPI in the absence of anionic phospholipids may be a more specific marker of thrombotic tendency in APS than aPL (4). Until now, high frequencies and levels of IgG anti-β_2GPI, comparable to those in autoimmune diseases, particularly systemic lupus erythematosus, have not been reported in non-autoimmune disease.

In this study we present the finding of a high frequency (42%) of IgG anti-β_2GPI in 45 children with AD. Furthermore, not only the high frequency but also the levels of IgG anti-β_2GPI were similar to those usually found in APS. Anti-β_2GPI was significantly associated with AD, as a low level of IgG anti-β_2GPI was detected in only one of 26 patients with other atopic diseases and in none of the 22 children who experienced a systemic anaphylactic reaction after stings of *Hymenoptera* insects.

Since the existence of anti-β_2GPI in AD children has not been associated with clinical manifestations of APS, we presumed that those antibodies may differ from anti-β_2GPI in APS patients. A series of experiments was performed to characterize the binding properties of anti-β_2GPI in AD in comparison with those in APS.

The specificity of anti-β_2GPI in AD was supported by the efficient inhibition of binding to solid-phase β_2GPI with β_2GPI in the fluid phase. Interestingly, >80% inhibition was obtained with 100 µg/ml of β_2GPI (Fig. 2), which was approximately half

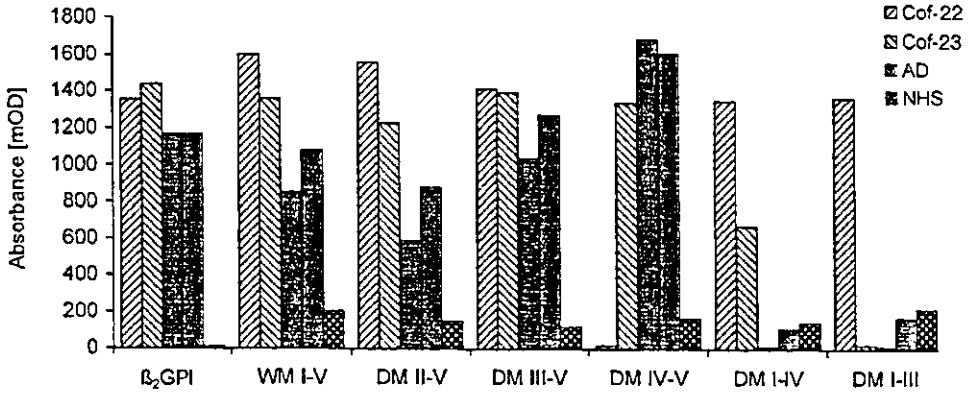

Fig. 4. Binding to serum-purified human β_2GPI (β_2GPI), whole molecule recombinant β_2GPI (WM) and various domain-deleted mutants of β_2GPI (DM). All nine tested sera from children with AD showed the same binding pattern and results of two sera are presented. Cof-22 mAb recognizing domain III (50 ng/ml) and Cof-23 mAb recognizing domain IV (100 ng/ml) were used as positive controls. A normal human serum (NHS) reaching the highest absorbance values is included for comparison. Results are presented as mOD (10^{-3} × OD) at 405 nm values.

the physiologic concentration in human serum (28). In contrast, anti-β_2GPI from the APS patients was very weakly inhibited by fluid-phase β_2GPI. This difference could be attributed to the presumed conformational specificity of anti-β_2GPI from APS patients, recognizing β_2GPI epitopes that are fully expressed only when β_2GPI is bound to an appropriate negatively charged surface, or to differing affinities for fluid-phase β_2GPI.

Another important distinction between anti-β_2GPI in AD and APS was noted using the standard and modified aCL ELISA. Namely, anti-β_2GPI from the children with AD showed negligible binding in either of these assays, indicating that their target epitopes were not available when β_2GPI (from FBS or purified human protein) was combined with cardiolipin. Therefore, anti-β_2GPI in AD could not be classified as aCL. Antibodies reacting in anti-β_2GPI, but not in aCL, ELISA were reported in some APS patients and healthy individuals, but were not further characterized (29,30).

The assays with deletion mutants of β_2GPI provided further evidence for a distinct fine specificity of anti-β_2GPI in AD. By contrast to antibodies from the APS patients, for which the major epitope proved to reside within domains I–IV (12–14), the sera from the children with AD showed a reactivity restricted to domain V of β_2GPI. Furthermore, a high inhibition of binding to β_2GPI was obtained by isolated domain V, confirming that this domain possessed main epitope(s) for anti-β_2GPI in AD.

Domain V of β_2GPI was shown to contain a potent phospholipid-binding site including the C-terminal loop (9). The proteolytic cleavage of the C-terminal loop between Lys317 and Thr318 by plasmin abolished the binding of anti-β_2GPI from the children with AD. The integrity of the phospholipid-binding site was also critical for the binding of anti-β_2GPI from APS patients in some (8,10,27,31), but not all studies (32). The lack of binding to cleaved β_2GPI was attributed to either a conformational change of β_2GPI (27) or direct disruption of the epitopic site in domain V (8,10,31). However, anti-β_2GPI from APS patients bound to β_2GPI combined with phospholipids (8,10,27,31), which was not the case for anti-β_2GPI in AD. The failure of anti-β_2GPI from children with AD to bind cleaved β_2GPI or β_2GPI associated with cardiolipin suggested that their epitopes were located at or at least in close vicinity to the phospholipid-binding site in domain V. We may also assume that a sterical hindrance caused by the binding of such antibodies to β_2GPI interferes with the attachment of β_2GPI to phospholipids, as already noted for Cof-18 mAb, which also recognizes domain V of β_2GPI (12). Since the adhesion of β_2GPI to negatively charged phospholipids and subsequent binding of anti-β_2GPI appears to be the pathogenic sequence of events in APS, the inability of AD-associated anti-β_2GPI to bind β_2GPI in complex with phospholipids may be an explanation for their presumed non-thrombogenicity.

The mechanisms responsible for the occurrence of IgG anti-β_2GPI in AD are not clear. Several interdependent factors are probably involved. In virtually all sera from children with AD having antibodies against human β_2GPI we found also a similar reactivity with bovine β_2GPI. However, a specific binding to bovine β_2GPI was also observed in some sera from children with atopic diseases without antibodies against human β_2GPI. The inhibition tests with human and bovine β_2GPI implied that children with AD harbored two subsets of anti-β_2GPI: one recognizing specifically bovine β_2GPI, found also in other atopic children, and the other cross-reactive with human and bovine β_2GPI, resembling anti-β_2GPI from APS patients in our study and previous reports (33). An important factor responsible for the occurrence of antibodies against bovine β_2GPI might be repeated exposure to nutritional β_2GPI early in life, when the intestinal mucosa is more permissive for large molecules (15). It is conceivable that in infancy the ingested bovine or some other type of β_2GPI (from dietary products) could act as a peroral immunization agent inducing a transient production of species-specific anti-β_2GPI. In children with AD this humoral immune response may spread also to human β_2GPI. An additional explanation for the development of antibodies against human β_2GPI in children with AD might also be related to a deficiency of a specific regulatory T cell function in AD (34).

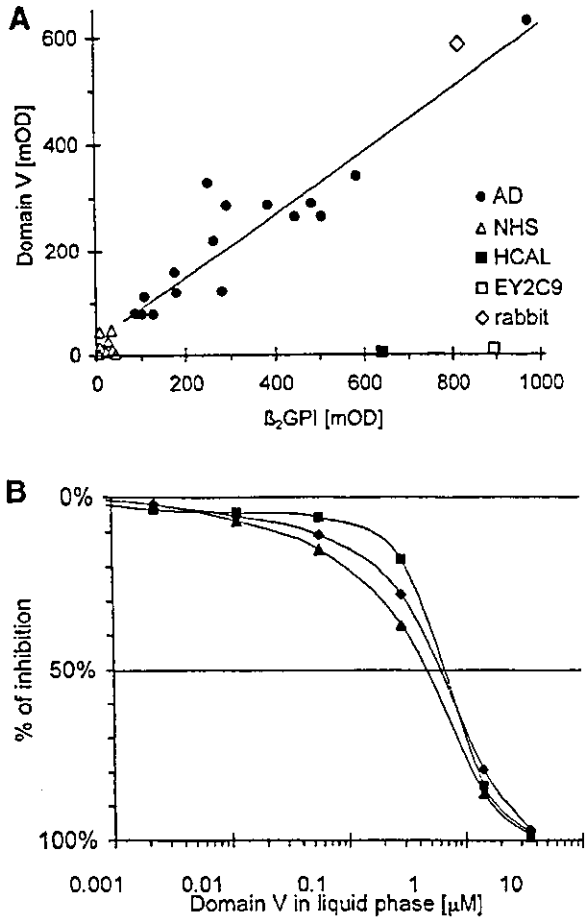

Fig. 5. (A) Binding of anti-β_2GPI to isolated domain V of β_2GPI. In 16 tested anti-β_2GPI$^+$ children with AD, a positive correlation was found between absorbance values for complete β_2GPI and domain V (r = 0.85, $P < 0.0001$). Rabbit polyclonal anti-β_2GPI (rabbit) was used as a positive control. HCAL and EY2C9 mAb recognizing domain IV, and normal human sera (NHS) were applied as negative controls. Results are presented as mOD (10^{-3} × OD) at 405 nm values. (B) Inhibition of binding of sera from children with atopic dermatitis to β_2GPI adsorbed on microtiter plates by domain V of β_2GPI in the fluid phase. Three representative inhibition profiles are presented. Results are expressed as percentage of inhibition (1 – $OD_{inhibited}$/$OD_{non\text{-}inhibited}$).

It has already been shown that exposure to cow milk during the first few months of life results in the initiation of immune responses towards β-lactoglobulin, BSA and α-casein. The specific IgG antibody production typically peaked during early infancy, with particularly high levels in children with AD, and declined thereafter (35,36). In our study as well, the AD children having IgG anti-β_2GPI were of a significantly younger age than those without anti-β_2GPI. Antibodies against BSA were also detected in our atopic children, particularly in AD. Although their frequency was similar to that of anti-β_2GPI, the potential cross-reactivity was excluded.

Anti-β_2GPI in children with AD were restricted to the IgG1 subclass. The IgG subclass distribution of antibodies reactive with dietary proteins, notably β-lactoglobulin, has already been investigated. In two studies, significantly higher levels of specific IgG1 and IgG4 were found in infants with elevated IgE. This parallelism in IgG1, IgG4 and IgE responses was ascribed to the influence of IL-4 (37,38). It is tempting to speculate that the elevated levels of IgG1 anti-β_2GPI in children with AD were also a result of a genetically governed T_h2 skewing of the response to new nutritional antigens in atopy prone infants.

At present we have no evidence that anti-β_2GPI in AD can influence allergic manifestations or induce APS. We are also not aware of any relation between AD and APS, considering patients' personal and family history.

In conclusion, we found a high frequency of IgG anti-β_2GPI in children with AD and no clinical signs of APS. These anti-β_2GPI could not be classified as classic aPL (aCL), as they did not bind to β_2GPI associated with cardiolipin. The proteolytic cleavage of the C-terminal region of β_2GPI in close vicinity to the phospholipid-binding site abolished the binding of anti-β_2GPI from children with AD, implying that the epitope resided near the phospholipid-binding region in domain V. Further analyses and prospective studies are needed for definite conclusions on the clinical relevance of anti-β_2GPI in AD. The investigation of interactions between dietary β_2GPI and the human immune system may provide insights into the regulation of autoimmune anti-β_2GPI response.

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Abbreviations

β_2GPI	β_2-glycoprotein I
aCL	anticardiolipin antibody
AD	atopic dermatitis
anti-β_2GPI	antibodies against human β_2GPI
aPL	antiphospholipid antibodies
APS	antiphospholipid syndrome
AU	arbitrary units
AUA	IgA AU
AUG	IgG AU
AUM	IgM AU
DM	recombinant domain-deleted mutant of human β_2GPI
WM	recombinant whole molecule β_2GPI

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Suppressed intrinsic fibrinolytic activity by monoclonal anti-beta-2 glycoprotein I autoantibodies: possible mechanism for thrombosis in patients with antiphospholipid syndrome

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Summary. β2-glycoprotein I (β2GPI) bears the epitope(s) for autoimmune anticardiolipin antibodies (aCL) frequently present in patients with antiphospholipid syndrome (APS). β2GPI is involved in coagulation and fibrinolytic systems, including inhibition of contact activation. Coagulation factor XII is an initiator of intrinsic coagulation and also of intrinsic fibrinolysis. We investigated the effect of aCL (= anti-β2GPI antibodies), regarding intrinsic fibrinolysis using autoimmune monoclonal anti-β2GPI antibodies derived from a patient with APS or from an NZW/BXSB-F1 mouse. We developed a chromogenic assay system to determine intrinsic fibrinolytic activity. The reaction was activated by kaolin in the euglobulin fraction. Exogenous β2GPI slightly suppressed intrinsic fibrinolytic activity of the euglobulin fraction from normal plasma. Human monoclonal anti-β2GPI antibody (EY2C9) and mouse monoclonal anti-β2GPI antibody (WBCAL-1) in the presence of β2GPI decreased the activity. In this system, the suppression remained significant in the presence of an excess of exogenous activated factor XII. Euglobulin fractions from APS patients' plasma paralleled low activities of intrinsic fibrinolysis compared with those from healthy subjects. Our results suggest that β2GPI and anti-β2GPI antibodies suppress intrinsic fibrinolytic activities. This suppression was not only due to inhibition of factor XII activation but was also related to function of activated factor XII (XIIa). These phenomena partly explain the mechanisms of thrombosis in APS.

Keywords: kaolin, chromogenic assay, antiphospholipid antibody, anticardiolipin antibody, fibrinolysis, anti-β2-glycoprotein I antibody.

The specificity of antiphospholipid antibodies (aPLs) associated with thrombotic events is not directed towards phospholipids but rather towards phospholipid binding proteins or phospholipid–protein complexes (Roubey, 1994). Antiphospholipid syndrome (APS), now recognized to be one of the most common causes of acquired thrombophilia (Hughes, 1993; Khamashta & Hughes, 1995), links thrombosis or pregnancy morbidity with the persistence of aPLs. Among phospholipid binding proteins, β2-glycoprotein I (β2GPI), which bears the epitope(s) for anticardiolipin antibody (aCL) binding, has been extensively studied. These epitope(s) are exposed when β2GPI binds to negatively charged phospholipids such as cardiolipin (Matsuura *et al*, 1994), thus behaving as a cofactor for aCL binding. β2GPI, formed by five short consensus repeat domains, is a 50-kDa phospholipid binding protein with a plasma concentration of about 200 μg/ml. The potent phospholipid binding site, consisting of the region K^{282}NKEKK^{287}, was found in the fifth domain of β2GPI (Sheng *et al*, 1996). Anticardiolipin antibodies recognized the epitope(s) in the absence of cardiolipin when β2GPI was coated onto polystyrene plates where oxygen was introduced by radiation (Matsuura *et al*, 1994), implying that aCL can bind not only to the cardiolipin–β2GPI complex but also to β2GPI alone. Therefore aCL with such characteristics are also currently termed anti-β2GPI antibodies (anti-β2GPI). Schousboe (1985) focused on the properties of β2GPI in the intrinsic pathway of coagulation and reported that β2GPI exerted inhibitory effects on this activity.

Factor XII, prekallikrein and high-molecular kininogen, all of which are important factors of the contact activation system, are also known to trigger intrinsic fibrinolysis, as well as intrinsic coagulation. The intrinsic fibrinolysis pathway has been primarily researched *in vitro* (Kluft *et al*,

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1987). This pathology is mediated by plasminogen pro-activator of blood, pro-urokinase and contact activation components. This pathway is possibly initiated in response to blood clotting, hence a primary preventable event related to thrombus formation. It is difficult, however, to investigate the effect of β2GPI and anti-β2GPI on intrinsic fibrinolysis as a system to determine intrinsic fibrinolytic activity has not been established. We developed a chromogenic assay system for the intrinsic fibrinolysis in euglobulin fractions, using kaolin as an activator. The effect of β2GPI and monoclonal anti-β2GPI autoantibodies, derived from lupus-prone mice or a patient with APS, on intrinsic fibrinolytic activity was explored. We also investigated the mechanism of thrombus formation in patients with APS, and the effects of β2GPI and antibodies on the activation of factor XII.

MATERIALS AND METHODS

Proteins. Human β2GPI was purified from normal sera, as described (Matsuura *et al.* 1992). The purity was confirmed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Fatty acid-free bovine albumin (BSA) was obtained from Sigma (catalogue number A-6002; MO, USA). An immunoglobulin (Ig)G mouse monoclonal anti-β2GPI, WBCAL-1, was derived from an NZW/BXSB-F1 mouse with myocardial infarction and a high titre of anti-β2GPI (Hashimoto *et al.* 1992). EY2C9 is an IgM human monoclonal anti-β2GPI established from an APS patient with a high titre of anti-β2GPI (Ichikawa *et al.* 1994). Characteristics of WBCAL-1 and EY2C9 have been reported (Hashimoto *et al.* 1992; Ichikawa *et al.* 1994). Briefly, both monoclonal antibodies bound to cardiolipin–β2GPI complex but not to cardiolipin alone. In the absence of cardiolipin, they did not recognize β2GPI immobilized on the standard enzyme-linked immunosorbent assay (ELISA) plate, but bound to β2GPI coated on an oxidized ELISA plate. Lupus anticoagulant activity was confirmed, using dilute Russell viper venom as an activator of coagulation factor X (Takeya *et al.* 1997). We considered that these characteristics represented autoimmune anti-β2GPI found in patients with APS. These monoclonal antibodies did not bind to factor XII coated on ELISA plates (data not shown). A control monoclonal IgM (TM1B9) lacked aCL activity (Ichikawa *et al.* 1994). The monoclonal antibodies, when purified from serum-free medium culture supernatant, showed a single band on agarose electrophoresis.

Polyclonal rabbit anti-β2GPI IgG was purchased from Cedarlane Laboratories (Ontario, Canada), and normal mouse IgG was from Caltag Laboratories (Burlingame, CA, USA). Prekallikrein-deficient (PKD) plasma, activated factor XII (XIIa) and phospholipid (PTT-Reagent RD®) were purchased from George King Bio-Medical (Kansas, USA), Enzyme Research Laboratories (Indiana, USA) and Roche Diagnostics (Switzerland) respectively.

Glu-plasminogen was purified from plasma of healthy donors by chromatography on lysine-Sepharose 4B (Pharmacia, Uppsala, Sweden) and diethyl aminoethyl (DEAE)-Sephadex A-50 (Pharmacia). The purity was confirmed by SDS-PAGE.

Preparation of platelet-free plasma. Blood samples were collected from 10 healthy donors by venepuncture using precooled plastic tubes that contained 0·105 mol/l citrate as anticoagulant (9:1, v/v). The samples were immediately centrifuged at 3000 r.p.m. for 15 min at 4°C and platelet-free plasma was prepared by passing through a 0·22 μmol/l pore size filter. Then the normal pooled plasma (PNP) was stored at −80°C until use.

Euglobulin fractions. The euglobulin fraction was prepared as described by Kluft (1979). Briefly, 1 volume of PNP was diluted with 9 volumes of cold distilled water, and acetate was added to adjust the solution to pH 5·9. After incubating at 4°C for 30 min, the solution was centrifuged at 3000 r.p.m. for 15 min. The pellet was resolved with 1 volume of EDTA buffer (50 mmol/l sodium diethlbarbiturate, 0·1 mol/l NaCl, 2·7 mmol/l EDTA, 0·25% gelatin, pH 7·8).

Assay for determination of intrinsic fibrinolytic activity in euglobulin fractions. To determine the activity of intrinsic fibrinolysis, we developed a new assay system, using the euglobulin fraction and a synthetic substrate S-2251 (Chromogenix AB, Möudal, Sweden), which is specific for plasmin activity. The sample, consisting of 50 μl of the euglobulin fraction prepared from PNP, 10 μl of phospholipid and 20 μl of either kaolin solution (2·5 mg/ml) or assay buffer A (50 mmol/l Tris-HCl, pH 8·8, 0·1 mol/l NaCl, 0·1 g/l TritonX100), was mixed with 100 μl of the assay reagent (0·6 μmol/l of Glu-plasminogen and 0·3 mmol/l of S-2251 in assay buffer A). After incubation at 37°C for 12 h in a shaded box, absorbance of the mixture was measured at 405 nm, using a plate reader (Titertek Multiskan MCC®; Absystems, Finland). The activity of intrinsic fibrinolysis was calculated by subtracting plasmin activity in the presence of kaolin from that in the absence of kaolin.

To establish the standard curve of intrinsic fibrinolytic activity, 12·5%, 25·0%, 50·0% and 100% of the euglobulin fraction from PNP diluted with EDTA buffer was mixed with phospholipid, either kaolin or assay buffer A, followed by the assay reagent. The activity of intrinsic fibrinolysis was then determined, as described above.

Effect of β2GPI and monoclonal anti-β2GPI on intrinsic fibrinolytic activity. To investigate the effect of β2GPI on intrinsic fibrinolytic activity, either β2GPI or BSA solution (final concentration 40·5, 81·5, 165·0 and 330·0 μg/ml) was added to solution, consisting of the diluted euglobulin fraction from PNP with the same volume of EDTA buffer (50% euglobulin fraction), the phospholipid with/without kaolin. The plasmin activity in each solution was determined, as described above.

The effect of monoclonal anti-β2GPI on activity of intrinsic fibrinolysis was investigated. The monoclonal anti-β2GPI (WBCAL-1: 5, 10 or 20 μg/ml; EY2C9: 10, 20 or 40 μg/ml) was mixed with solution consisting of the 50% euglobulin fraction, β2GPI (160 μg/ml) and phospholipid, with/without 2·5 mg/ml kaolin. The reagent was added to the mixture and the activity of intrinsic fibrinolysis was measured, as described above.

In addition, the β2GPI dependency on the effect of WBCAL-1 or EY2C9 was also investigated. The euglobulin

fraction was prepared from the plasma of an individual with homozygous β2GPI deficiency (β2GPI-Sapporo) (Yasuda *et al*, 2000). The effect of WBCAL-1 and EY2C9 on intrinsic fibrinolysis was evaluated using complete β2GPI-deficient euglobulin fraction, in the same fashion in the presence/absence of exogenous β2GPI.

Effect of β2GPI and monoclonal anti-β2GPI on the activation of factor XII. As one of the triggers for intrinsic fibrinolysis is activation of coagulation factor XII, the effect of β2GPI and/or WBCAL-1 on this activation was investigated. The assay system used for determination of factor XIIa, which makes use of PKD plasma and a synthetic substrate S-2302 (Chromogenix AB), a substrate for factor XIIa and kallikrein, was developed according to Schousboe and Rasmussen (1995) with minor modification. An aliquot (30 μl) of sample solution, consisting of IgG (WBCAL-1, normal mouse IgG or polyclonal rabbit anti-β2GPI IgG: 0, 20 or 40 μg/ml), and 10 μl phospholipid with/without β2GPI (90 μg/ml) were incubated for 20 min at 37°C, and then mixed with 60 μl of assay buffer B (50 mmol/l Tris-HCl, pH 8·8, 0·1 mol/l NaCl, 1mmol/l CaCl2, 0·1 g/l TritonX100) and 10 μl of PKD plasma, with/without 10 μl of 2·5 mg/ml kaolin. After incubating at 37°C for 20 min, 50 μl of the reagent, composed of S-2302 (0·97 mmol/l), 30 mmol/l EDTA and buffer C (0·1 mol/l Tris-HCl, pH 8·8, 0·2 mol/l NaCl, 0·2 g/l Triton-X100), was added to the mixture. After further incubation at 37°C for 90 min, OD 405 nm of the mixture was examined. The activity of factor XIIa converted from factor XII by the addition of kaolin was calculated, by subtracting OD of the sample in the absence of kaolin.

Effect of monoclonal anti-β2GPI on intrinsic fibrinolytic activity in the presence of factor XIIa. To investigate the effects of monoclonal antibodies on intrinsic fibrinolysis, which depend on steps of factor XII activation, an excess of exogenous factor XIIa was added to the intrinsic fibrinolytic activity assay. WBCAL-1 was added in the intrinsic fibrinolytic activity assay in the presence of factor XIIa (6 μg/ml).

Intrinsic fibrinolytic activity in euglobulin fractions of plasma from patients with APS. The intrinsic fibrinolytic activities of patients with APS, as initiated by kaolin, were compared with healthy subjects. The euglobulin fractions from plasma of 14 APS patients with high titres of anti-β2GPI (two men and 12 women, 42·5 ± 12·3 years) and 18 healthy subjects (10 men and eight women, 28·2 ± 4·3 years) were prepared, as described above. These euglobulin fractions were incubated in the presence of 160 μg/ml β2GPI, phospholipid with/without kaolin, and the assay reagent (0·6 μmol/l of Glu-plasminogen and 0·3 mmol/l of S-2251 in assay buffer A) at 37°C for 12 h, followed by OD 405 nm determination.

Statistical analysis. Data are expressed as mean ± SD. Differences between data sets were analysed using the unpaired Student's *t*-test, and differences between the activity index (AI) of APS patients' euglobulin and control subjects were analysed using the Mann–Whitney non-parametric test. A *P*-value of $< 0{\cdot}05$ was considered to be statistically significant.

RESULTS

Assay system for intrinsic fibrinolytic activity

The euglobulin fraction contained 7·6%, 17·6%, 60·6%, 20·2% and 13·3% of plasminogen, prekallikrein, factor XI, factor XII and β2GPI, respectively, when compared with PNP.

Figure 1 shows the intrinsic fibrinolytic activity in euglobulin fractions from PNP. In all evaluations, 50% of the euglobulin fraction was used. The calculation

OD405 with kaolin − OD405 without kaolin

was expressed as an activity index (AI): 0·25, 0·5, 1·0 and 2·0 AI, corresponding to the intrinsic fibrinolytic activity obtained from 12·5% 25·0%, 50·0% and 100% PNP euglobulin fraction respectively ($n = 4$, all in triplicate).

Effect of β2GPI and monoclonal anti-β2GPI on intrinsic fibrinolytic activity

Different concentrations of purified β2GPI were added to the intrinsic fibrinolytic activity assay. Intrinsic fibrinolytic activity was significantly suppressed by adding β2GPI, compared with that of BSA (Fig 2) ($n = 4$, all in triplicate).

When the mouse monoclonal anti-β2GPI, WBCAL-1, was added to the intrinsic fibrinolytic activity assay in the presence of β2GPI (160 μg/ml), the activity with WBCAL-1 was significantly suppressed (Fig 3A) compared with use of the same concentration of control IgG.

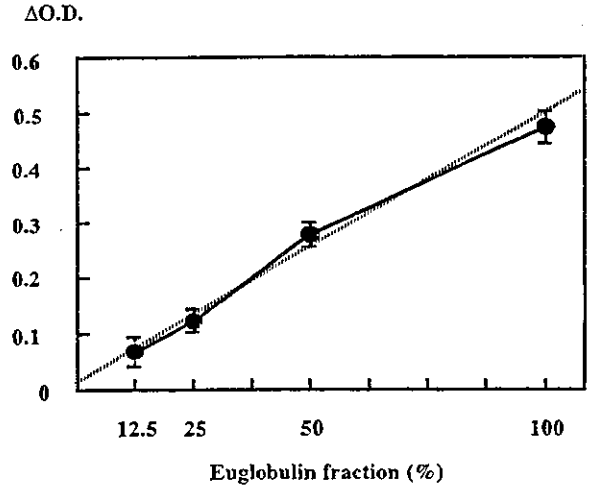

Fig 1. Intrinsic fibrinolytic activity assay and the standard curve. Samples containing 12·5%, 25·0%, 50·0%, 100% of the euglobulin fraction were incubated with phospholipid, plasminogen and S-2251 in the presence or absence of kaolin, as described in *Materials and methods*. The activity of intrinsic fibrinolysis was calculated by subtracting plasmin activity in the presence of kaolin from that in the absence of kaolin, and expressed as an activity index (AI): 0·25, 0·5, 1·0 and 2·0 AI, corresponding to the intrinsic fibrinolytic activity obtained from 12·5%, 25%, 50% and 100% PNP euglobulin fraction, respectively ($n = 4$, all in triplicate). The standard curve is shown in this figure with the *Y*-axis representing OD values that correspond to PNP euglobulin concentrations.

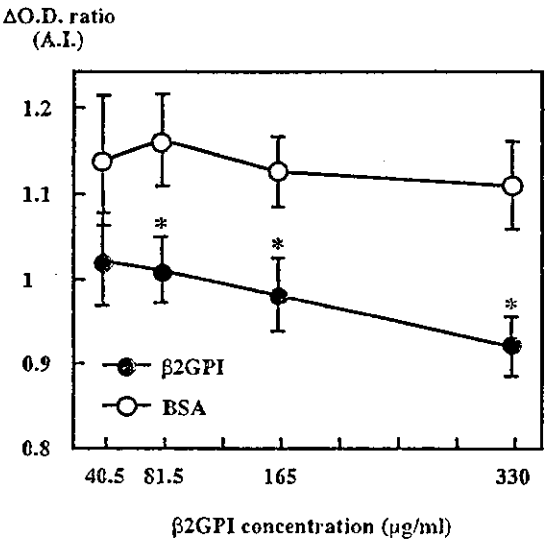

Fig 2. Effects of β2GPI on intrinsic fibrinolytic activity. Different concentrations of β2GPI were added to the intrinsic fibrinolytic activity assay. BSA was used as a control protein. β2GPI suppressed intrinsic fibrinolytic activity, in a dose-dependent manner (closed circles), compared with no effect of BSA (open circles) in this system. (*$P < 0.05$, compared with a control) ($n = 4$, all in triplicate).

EY2C9, a human monoclonal IgM anti-β2GPI, also markedly downregulated intrinsic fibrinolytic activity in the presence of β2GPI. In contrast, no effect of control monoclonal IgM was evident (Fig 3B).

In the study of the intrinsic fibrinolytic activity assay using complete β2GPI-deficient euglobulin fraction, neither WBCAL-1 (Fig 3C) nor EY2C9 (data not shown) inhibited activity in the absence of exogenous β2GPI. All experiments in Fig 3 were done in triplicate and confirmed by each repetition.

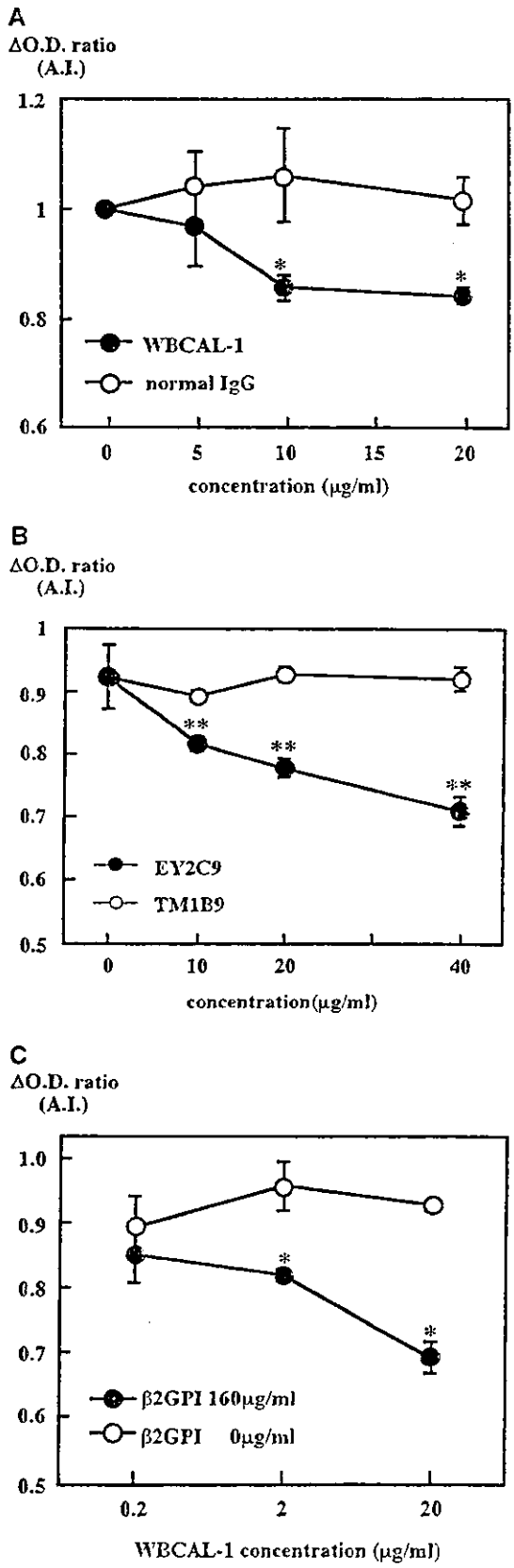

Fig 3. Effects of monoclonal antiβ2GPI autoantibodies on intrinsic fibrinolytic activity. The effects of monoclonal antiβ2GPI on the activity of intrinsic fibrinolysis were investigated. (A) WBCAL-1, mouse monoclonal antiβ2GPI (5, 10 and 20 μg/ml), was added to the intrinsic fibrinolytic activity assay in the presence of β2GPI (160 μg/ml). WBCAL-1 (closed circle) significantly suppressed the intrinsic fibrinolytic activity in a concentration-dependent manner, compared with effects of normal mouse IgG (open circle). (B) A human monoclonal antiβ2GPI derived from a patient with APS, EY2C9, was added into the intrinsic fibrinolytic activity assay (10, 20 and 40 μg/ml) in the presence of β2GPI. EY2C9 markedly suppressed the activity (closed circles) in contrast with no effect of TM1B9 (open circles), a control human monoclonal IgM, on this system. (C) β2GPI dependency on the inhibiting effect of WBCAL-1 on the intrinsic fibrinolytic activity assay. The β2GPI-free euglobulin fraction was prepared from plasma of an individual with congenital β2GPI deficiency. The intrinsic fibrinolytic activity assay was carried out with β2GPI-deficient euglobulin in the presence or absence of exogenous β2GPI (160 μg/ml). WBCAL-1 (0·2, 2 and 20 μg/ml) was added to the system. WBCAL-1 suppressed the intrinsic fibrinolytic activity in the presence of β2GPI (closed circles), but not in its absence (open circles) (*$P < 0.05$, **$P < 0.01$, compared with a control). All experiments were done in triplicate and confirmed by each repetition.

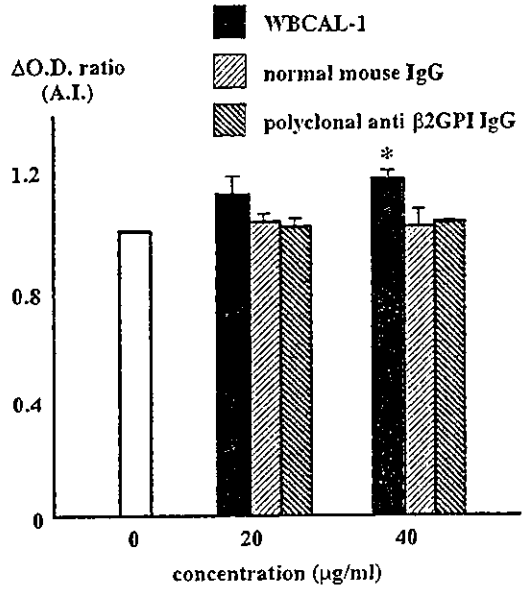

Fig 4. Effects of WBCAL-1 on factor XII activation. In PKD plasma with 90 μg/ml of β2GPI, WBCAL-1, normal mouse IgG or IgG fraction of polyclonal anti-β2GPI was added in the presence or absence of kaolin, and activated factor XII was measured, as described in *Materials and methods*. WBCAL-1 (closed bar) increased the factor XIIa generation, thereby implying that WBCAL-1 reduced inhibition of factor XIIa generation by β2GPI. However, normal mouse IgG did not affect factor XIIa generation and polyclonal anti-β2GPI did not exert any influence on factor XIIa generation (*$P < 0.05$, compared with a control) ($n = 3$, all in triplicate).

Effect of β2GPI and monoclonal anti-β2GPI on activation of factor XII

When the effect of β2GPI on activation of factor XII was investigated using PKD plasma and synthetic substrate S-2302, factor XII activation in PKD plasma was significantly reduced by over 45 μg/ml of β2GPI (data not shown). When different concentrations of WBCAL-1 were added to the factor XII activation assay in the presence of 90 μg/ml β2GPI, WBCAL-1 either did not affect or reduced the inhibition by β2GPI (Fig 4) ($n = 3$, all in triplicate). Factor XII activation was not affected by polyclonal anti-human β2GPI antibodies and normal mouse IgG.

Effect of monoclonal anti-β2GPI on intrinsic fibrinolytic activity in the presence of factor XIIa

When 20 μg/ml of WBCAL-1 was added to the intrinsic fibrinolytic activity assay in the presence of factor XIIa (6 μg/ml), the reduction in intrinsic fibrinolysis was maintained (WBCAL-1 0 μg/ml *vs* 20 μg/ml; AI 0·99 ± 0·011 *vs* 0·71 ± 0·032 respectively, $P < 0.05$).

Activities of intrinsic fibrinolysis in the euglobulin fractions prepared from plasma of patients with APS

Euglobulin fractions were prepared from 14 patients with APS and 18 control subjects. The AI in the intrinsic fibrinolytic activity assay in patients with APS was significantly lower compared with those in control subjects.

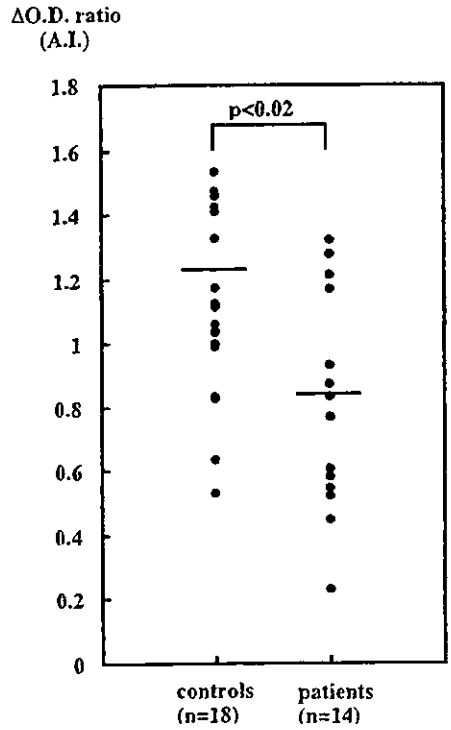

Fig 5. Intrinsic fibrinolytic activity in APS patients. The intrinsic fibrinolytic activities of euglobulin fractions from APS patients ($n = 14$) and healthy control subjects ($n = 18$) in the presence of 160 μg/ml of β2GPI. These euglobulin fractions prepared from patients showed a significantly lower intrinsic fibrinolytic activity than those seen from control subjects.

Distribution of intrinsic fibrinolytic activities in those euglobulins is shown in Fig 5.

DISCUSSION

We developed a new assay system that can evaluate intrinsic fibrinolytic activity in the euglobulin fraction with the synthetic substrate S-2251. The subtraction of fibrinolytic activity in the absence of kaolin from that in its presence was defined as intrinsic fibrinolytic activity in the plasminogen-enriched euglobulin fraction. After dextran sulphate precipitation, kaolin did not work as the activator of fibrinolysis. Further, the euglobulin fraction, prepared by factor XII or prekallikrein-deficient plasma, did not display fibrinolysis activation by kaolin (data not shown). These results suggested that our system represented intrinsic fibrinolysis activity. We investigated the effect of β2GPI or anti-β2GPI on intrinsic fibrinolytic activity in the euglobulin fractions, using our new method. As β2GPI has potent binding properties to negatively charged proteins or to phospholipids involved in coagulation processes, it is likely that aCL (anti-β2GPI) interact in the steps of β2GPI-associated coagulation. Although the physiological role of β2GPI remains uncertain, a number of studies have demonstrated the *in vitro* function of β2GPI. β2GPI inhibits

ADP-induced platelet aggregation (Nimpf *et al*, 1985), prothrombinase activity (Nimpf *et al*, 1986), factor Va degradation by activated protein C (Mori *et al*, 1996) and factor Xa generation (Shi *et al*, 1993). Bancsi *et al* (1992) and our team (Yasuda *et al*, 2000) reported that β2GPI-deficient families were apparently not at risk of thrombosis. We have also reported that most haemostatic and fibrinolytic markers are normal with any evidence of increased thrombin generation in individuals with congenital β2GPI deficiency (Takeuchi *et al*, 2000). Moreover, plasma β2GPI levels in patients with APS have been reported to be either normal (de Benedetti *et al*, 1992) or elevated (Galli *et al*, 1992; McNally *et al*, 1995). Therefore, mechanisms related to thrombosis in patients with anti-β2GPI cannot be explained as the result of a secondary β2GPI deficiency, but it seems likely that anti-β2GPI modify the physiological roles of β2GPI. It has been shown that anti-β2GPI increases the affinity between β2GPI and phospholipids, thus bivalency of IgG anti-β2GPI may be crucial for their roles in modifying β2GPI properties (Arnout *et al*, 1998). Effects of β2GPI and immunized antibodies to β2GPI on the contact activation system were reported (Schousboe & Rasmussen, 1995). In purified systems, the inhibition of factor XII activation (Henry *et al*, 1988) was found to be responsible. β2GPI inhibited the activation of factor XIIa-dependent prekallikrein (Schousboe, 1988). We have obtained evidence that β2GPI inhibited intrinsic fibrinolytic activity, as determined using our new chromogenic assay for fibrinolytic activity. The presence of monoclonal antibodies to β2GPI resulted in the inhibition of fibrinolytic activity. The manner in which plasminogen is activated by this pathway was not clear, however, this inhibition of fibrinolysis was attributed to a reduced contact activation reaction initiated by coagulation factor XII activation. Our study is the first report to show that autoimmune monoclonal anti-β2GPI downregulated the intrinsic fibrinolysis system. Anti-β2GPI functioned in the system in a β2GPI-dependent fashion. Schousboe and Rasmussen (1995) reported that β2GPI inhibited factor XII activation and that immunized polyclonal anti-β2GPI enhanced this inhibition. Our results also showed that β2GPI slightly inhibited the intrinsic fibrinolysis system. However, behaviour of anti-β2GPI autoantibodies and anti-β2GPI-immunized antibodies did not coincide with respect to the steps of factor XII activation. Monoclonal autoantibodies did not enhance inhibitory functions of β2GPI in factor XII activation, rather they neutralized it.

The immunological properties and epitope distribution differ between autoimmune and immunized anti-β2GPI. The epitope exposure of β2GPI, presumably located on domain IV (Igarashi *et al*, 1996) of β2GPI, for autoimmune anti-β2GPI depends on interaction with phospholipids, i.e. autoimmune anti-β2GPI does not recognize native β2GPI but does recognize the β2GPI–phospholipids complex (Matsuura *et al*, 1998; Koike *et al*, 1999). On the other hand, immunized anti-β2GPI binds to β2GPI without any alteration in conformation. Those differences in the characteristics may account for the heterogeneity of the effect on intrinsic fibrinolysis. Our results showed that the target reaction of this inhibition by β2GPI and monoclonal antibodies was not activation of factor XII. The reaction following activation of factor XII, such as the activation of prekallikrein by factor XIIa, has to be considered. It was reported that prekallikrein was activated by factor XIIa on the surface of platelets (Gurewich *et al*, 1993), and kallikrein converted from prekallikrein can activate pro-urokinase (single chain-urokinase type plasminogen activator: scu-PA) (Ichinose *et al*, 1986) in the presence of high-molecular kininogen (Mandle *et al*, 1976). Two-chain urokinase-type plasminogen activator (tcu-PA), converted from scu-PA, can activate plasminogen to plasmin on platelets or fibrin, and plasmin further converts scu-PA to tcu-PA. Thus, effective fibrinolysis may be the result of activation of prekallikrein. β2GPI may inhibit the activation of prekallikrein or the activation of pro-urokinase by kallikrein. Monoclonal anti-β2GPI monoclonal antibodies can inhibit the activity of intrinsic fibrinolysis in the presence of β2GPI.

The potential disorder of intrinsic fibrinolysis in patients with APS, which was determined by use of the euglobulin fractions, was suggested, although patient IgG may direct to multiple antigens, including factor XII. This decrease was smaller than expected, considering the results of the investigations on euglobulin fraction from PNP, β2GPI and monoclonal anti-β2GPI. This difference may be due to the smaller concentration of IgG because the euglobulin fraction contained only 6·3% IgG of the original plasma (unpublished observation).

Regarding the pathophysiology of thrombosis in APS, a number of mechanisms have been proposed, but have not been fully clarified. We consider that the mechanism is complex and that many mechanisms involving anti-β2GPI may contribute to the development of thrombosis in patients with aPLs. For example, anti-β2GPI can induce procoagulant substances on peripheral blood mononuclear cells or on endothelial cells (Smirnov *et al*, 1995; Del PaPa *et al*, 1997; Amengual *et al*, 1998; Atsumi *et al*, 1998a). The presence of anti-β2GPI results in inhibition of protein C anticoagulant activity (Atsumi *et al*, 1998b; Ieko *et al*, 1999). In addition to these mechanisms, the inhibition of intrinsic fibrinolysis by anti-β2GPI in the presence of β2GPI may play an important role in the thrombogenic state in APS, although it has been shown only as *in vitro* phenomena.

In conclusion, the intrinsic fibrinolysis activity assay established in this study was shown to be a useful method to investigate the effects of monoclonal anti-β2GPI on intrinsic fibrinolysis. Impaired intrinsic fibrinolysis caused by aPLs may be one of the important mechanisms related to thrombotic tendencies in patients with APS, and their further clarification will ultimately lead to the better management of the affected patients.

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