

# ACTIVATION OF ENDOTHELIAL CELLS BY ANTIPHOSPHOLIPID ANTIBODIES—A POSSIBLE MECHANISM TRIGGERING THROMBOSIS IN PATIENTS WITH ANTIPHOSPHOLIPID SYNDROME

Pei-Pei Chen, Yu-Chih Lin, Kun-Che Wu,<sup>1</sup> Jeng-Hsien Yen, Tsan-Teng Ou,  
Chen-Ching Wu, Hong-Wen Liu, and Wen-Chan Tsai

Division of Rheumatology, Department of Internal Medicine, Kaohsiung Medical University Hospital, and <sup>1</sup>Wu Kun Che Gynecologic, Obstetric and Pediatric Hospital, Kaohsiung, Taiwan.

Antiphospholipid syndrome (APS) is an antibody-mediated hypercoagulable state characterized by recurrent venous and arterial thromboembolic events. The presence of serum antibodies are collectively termed as antiphospholipid antibodies (aPL) and is the hallmark of the disease. Interest in the pathogenesis has mostly been focused on the blood coagulation factor. However, endothelial cells might play an important role. When stimulated, cell membrane would flip to expose negatively charged phospholipids and activation markers such as adhesive molecules may appear. We consider that these changes may play an important role in the initiation of the thrombotic process when endothelial cells encounter aPL. In this study, we incubated human umbilical vein endothelial cells (HUVECs) with IgG isolated from patients with APS and found that the HUVECs were activated by the expression of negatively charged phospholipids, as shown by high annexin V binding and negative propidium iodide staining and by an increase in the level of intracellular cell adhesion molecule-1 on the cell surface. The above findings indicate that endothelial cells can be activated on exposure to aPL and trigger the thrombotic event.

**Key Words:** antiphospholipid antibodies, human umbilical vein endothelial cells  
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Antiphospholipid syndrome (APS) is a disease characterized by the hypercoagulable state with clinical manifestation of venous and arterial thrombosis and fetal loss [1,2]. The diagnosis is made according to clinical findings and one of the two laboratory criteria including positive anticardiolipin antibody (aCL) and lupus anticoagulant (LA) [3]. Nevertheless, several observations indicate that the coagulation system is triggered, as shown by the inhibition of physiologic

anticoagulants and the inhibition of fibrinolysis [4–7]. The inhibition of both protein C activation by thrombin and thrombomodulin and of proteolytic cleavage of factor Va and factor VIIIa by activated protein C has been observed in association with APS [8–10]. Autoantibodies to vascular heparin sulfate proteoglycan can block the activation of natural anticoagulant antithrombin III [11–13]. In addition, there is some evidence of increased plasminogen activator inhibitor-1 [14] and the autoantibody on factor XII-dependent fibrinolysis [15,16].

Under resting conditions, endothelial cells exhibit the property of an anticoagulant, which keeps vessels patent with uncoagulated blood. After vascular damage or inflammation, endothelial cells play an important role in triggering the coagulation system.

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Address correspondence and reprint requests to: Dr Wen-Chan Tsai, Division of Rheumatology, Department of Internal Medicine, Kaohsiung Medical University Hospital, 100 Tzyou 1<sup>st</sup> Road, Kaohsiung 807, Taiwan.  
E-mail: d10153@ms14.hinet.net

Despite much attention being focused on the blood components of the coagulation system in APS patients, we think that in the presence of antiphospholipid antibodies (aPL), endothelial cells may show some changes to unclot the anticoagulant state and promote the process of hypercoagulation.

## MATERIALS AND METHODS

### Patients

Five patients from outpatient clinics of the Rheumatology Division, Kaohsiung Medical University Hospital, who fulfilled classification criteria for APS (Sapporo criteria [3]) were enrolled in the study. All patients were either positive for LA, aCL or anti- $\beta$ 2-glycoprotein-1 ( $\beta$ 2-GP-1). The age of the five patients ranged from 20 to 44 years (mean, 27 years) (Table 1). This study was approved by the medical ethics committee of Kaohsiung Medical University Hospital, and all patients signed a consent form.

### Purification of IgG

Immunoglobulin G (IgG) was isolated from the sera of APS patients by protein G sepharose (Amersham, Uppsala, Sweden). The protein concentration of the purified antibodies was determined by Bradford assay and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The isolated IgG was further concentrated by the use of Amicon Ultra-4 (Millipore Corp., Billerica, MA, USA) with centrifugation at 4000 g for 10 minutes. The concentrate was then filtered by 0.22  $\mu$ m microporous membrane (Millipore Corp.) to make it sterile.

### Cells

Human umbilical vein endothelial cells (HUVECs) were isolated from the umbilical veins of normal term

deliveries as described previously [17]. Briefly, the vein was rinsed with phosphate-buffered saline (PBS) and then with Hank's balanced salt solution (Sigma Chemical Co., St Louis, MO, USA) through 19-gauge drawing-up needles secured into the vein. Then, 0.1% collagenase Type IV (Sigma Chemical Co.) was introduced into the umbilical vein and the cord was submerged in warm cord buffer and incubated at 37°C in a humidified 5% CO<sub>2</sub> at atmosphere for 15 minutes. The collagenase solution containing HUVECs was aspirated from the vein, and centrifuged twice at 1,000 rpm for 5 minutes. HUVECs contained in this pellet were resuspended in Iscove's Modified Dulbecco's Medium (Biological Industries Ltd., Kibbutz Beit Haemek, Israel) containing 10  $\mu$ g/mL of endothelial cell growth supplement (Upstate Biotechnology Inc., Lake Placid, NY, USA), 10% fetal calf serum (Biological Industries Ltd.), 10  $\mu$ g/mL of insulin (Sigma Chemical Co.), and 33 mM 3-isobutyl-1-methylxanthine (Sigma Chemical Co.), 0.1% antibiotics (penicillin, streptomycin, amphotericin). HUVECs were then seeded into a sterile 75 cm<sup>2</sup> culture flask (NUNC A/S, Roskilde, Denmark) that had been coated with 0.8% gelatin (Merck, Darmstadt, Germany). The culture medium was changed every other day and the culture continued in a humidified 5% CO<sub>2</sub> atmosphere until 80% confluence was reached. HUVECs were only used from passages 2–3 in this study.

### Flow cytometry

HUVECs were seeded at 4  $\times$  10<sup>5</sup> cells/mL density on 48-well plates (Costar/Corning, Acton, MA, USA) for flow cytometry. After the 6-hour treatment of APS IgG (1 mg/mL), cells were harvested and washed in PBS. After resuspension in 100  $\mu$ L of annexin V binding buffer (10 mmol/L HEPES, 140 mmol/L NaCl, 2.5 mmol/L CaCl<sub>2</sub>, pH 7.4; Molecular Probes Inc.,

**Table 1.** Clinical and laboratory data in five antiphospholipid syndrome patients

Patient	Sex	Age (yr)	Clinical diagnosis	aPL test
1	F	24	SLE, CVA (artery thrombosis)	aCL(+/-), LA(+), anti- $\beta$ 2-GP-1(+)
2	F	31	Vasculitis, abortion	aCL(+/-), LA(+), anti- $\beta$ 2-GP-1(+)
3	F	44	SLE, venous thrombosis	aCL(+)
4	F	20	MCTD, artery thrombosis	aCL(+)
5	F	30	SLE, abortion	LA(+), anti- $\beta$ 2-GP-1(+)

aPL = antiphospholipid antibody; SLE = systemic lupus erythematosus; CVA = cerebrovascular accident; aCL = anticardiolipin antibody; LA = lupus anticoagulant; anti- $\beta$ 2-GP-1 = anti- $\beta$ 2-glycoprotein-1; MCTD = mixed connective tissue disease.

Eugene, OR, USA), samples were stained with 2  $\mu$ L of annexin V–fluorescein isothiocyanate (FITC) (Strong Biotech Corp., Taiwan) together with 1 mg/mL of propidium iodide, and incubated for 15 minutes. Intracellular cell adhesion molecule-1 (ICAM-1) was detected by adding 2  $\mu$ L phycoerythrin (PE) conjugate ICAM-1 (CD54; BioLegend Inc., San Diego, CA, USA) for 30 minutes. Analysis was performed on a FACScan

flow cytometry (Partec GmbH, Münster, Germany) equipped with FloMax software. For each sample, 10,000 events were collected and mean fluorescence intensity (MFI) was analyzed (Table 2).

## RESULTS

### Changes in cell membrane phospholipids

When HUVECs were cultured in the presence of IgG isolated from the five patients, the cell membrane expressed more negatively charged phospholipids in their outer layers, which was represented by stronger annexin V binding. In addition, the negative staining of the cells by propidium iodide indicated that the nuclear membranes were still intact (Figure 1, Table 2).

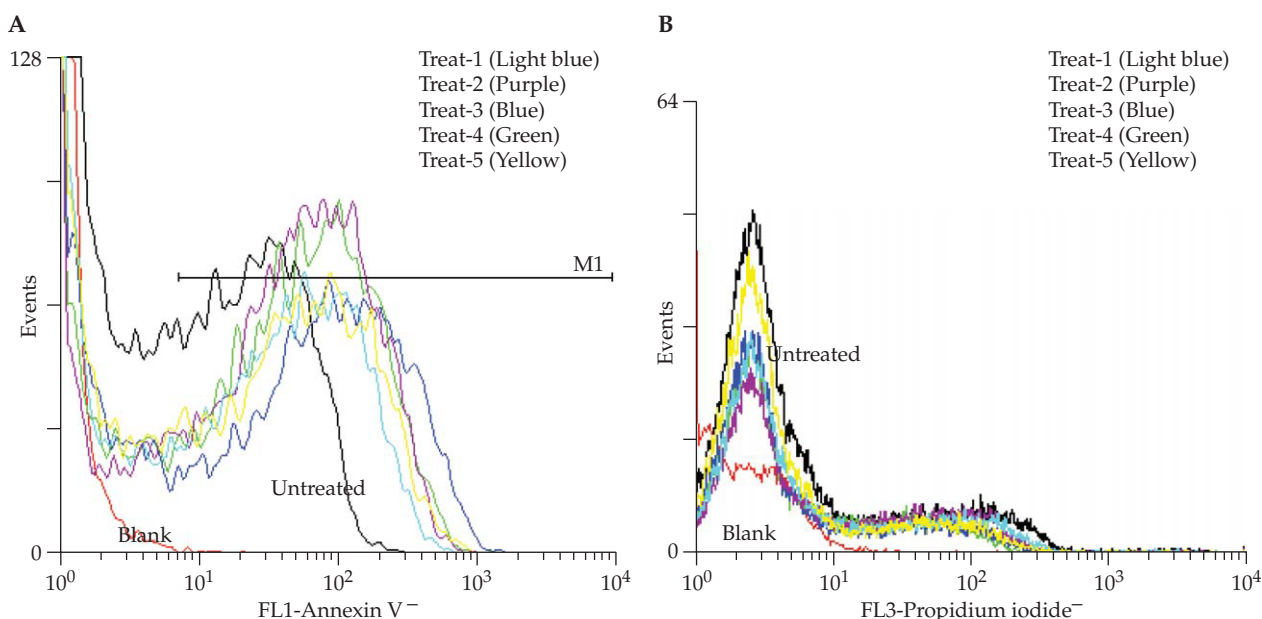
### Expression of adhesive molecules

To test whether the endothelial cells were activated by the antibodies, we further analyzed the expression of the adhesion molecule ICAM-1 on the cell surface when the cells were exposed to IgG isolated from the patients. All five IgGs isolated from the patients increased the surface expression of ICAM-1 (Figure 2, Table 2).

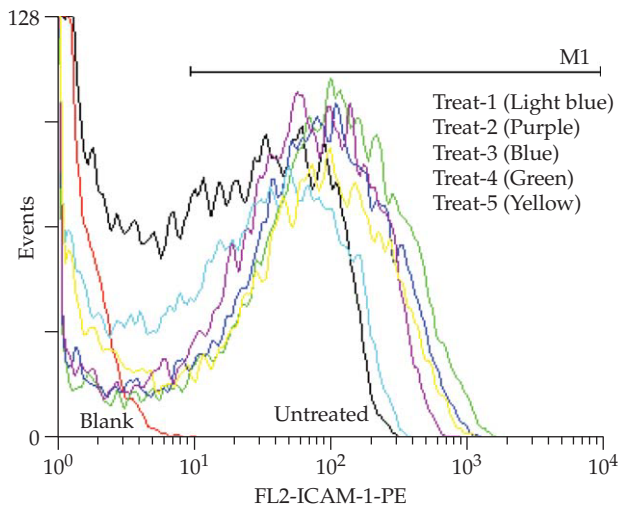
**Table 2.** Expression of annexin V and intracellular cell adhesion molecule-1 (ICAM-1) and cell viability of human umbilical vein endothelial cells after incubation with antiphospholipid antibody-containing immunoglobulin G purified from five patients with antiphospholipid syndrome

	Annexin V		PI	ICAM-1	
	MFI	%		MFI	%
Untreated	1.65	32.94	0.26	0.74	17.95
Patient 1	4.81	51.60	3.31	1.40	32.96
Patient 2	7.13	67.26	0.26	5.63	66.98
Patient 3	9.65	55.36	0.24	8.70	72.08
Patient 4	6.71	52.27	0.25	13.27	52.99
Patient 5	5.86	53.74	0.26	7.56	59.87

PI = propidium iodide; MFI = mean fluorescence intensity; % = percentage of positive cells.



**Figure 1.** Effects of antiphospholipid antibody-containing immunoglobulin G (IgG) purified from antiphospholipid syndrome (APS) patients on: (A) annexin V expression; and (B) cell viability detected by flow cytometry. (A) After incubation of human umbilical vein endothelial cells with IgG isolated from five patients with APS, the expression of negatively charged phospholipids on the cell surface increased, which was detected by increased annexin V–fluorescein isothiocyanate binding. (B) At the same time, the intact nuclear membrane, detected by propidium iodide, denotes that the cells are still alive.



**Figure 2.** Effect of antiphospholipid antibody-containing immunoglobulin G (IgG) purified from antiphospholipid syndrome (APS) patients on intracellular cell adhesion molecule-1 (ICAM-1) expression of human umbilical vein endothelial cells (HUVECs) detected by flow cytometry. After incubation of HUVECs with IgG isolated from five patients with APS, the expression of adhesion molecule ICAM-1 on the cell surface increased, as detected by increased anti-ICAM-1-phycoerythrin (PE) binding. The black line is the negative control.

## DISCUSSION

APS is characterized by hypercoagulation. Besides the influence of antibodies on the blood components including coagulation factors, cofactors, protein C and protein S have been reported in many papers [18,19]. We thought that antibodies might trigger the procoagulant components of endothelial cells and hence start the coagulation cascade. Our study found that when exposed to IgGs, the cell membrane began to increase the expression of negatively charged phospholipid on their outer layers. There are two possibilities when a cell has increased expression of negatively charged phospholipids on the cell membrane but has an intact nuclear membrane: apoptosis and cell activation. When apoptosis begins, the first sign is cell membrane flipping the inner layer of phospholipid bilayers out [20]. When the condition deteriorates, the cell membrane would further disrupt and encircle some cytoplasmic content to produce apoptotic bodies. On the other hand, when endothelial cells are activated, they express adhesive molecules for further interaction with intravascular

cells such as white blood cells and platelets [18]. In our experiment, the increased expression of ICAM-1 on HUVECs in addition to membrane flipping indicated that the cells were activated and not in an apoptotic state. However, the possibility exists that after cell activation, the endothelial cell may progress to cell death, the so-called “activation-induced cell death”. No matter what happens after cell activation, negatively charged phospholipids are already exposed, and the increased negative charge on the cell surface is the key factor initiating the intrinsic pathway of the coagulation system, and the high affinity of  $\beta 2$ -GP-1 to negatively charged phospholipids is considered to be the triggering mechanism for the development of anti- $\beta 2$ -GP-1, which is specific for autoimmune disease associated APS [21,22].

There are a variety of aPL. The epitopes of target antigens vary among different clones of antibodies. Some patients' antibodies are reactive only with cardiolipin, while others react with reagents of Venereal Disease Research Laboratory, LA, and even with some coagulation factors [23–25]. Antibodies are not always cross-reactive. Based on high heterogeneity of antigens, it is not possible to purify these antibodies by using a single antigen such as cardiolipin. As in many other reports, we used purified IgG from APS patients as the source of aPL [26–28]. The limitation of our study is that no healthy control serum or IgG was used. In another experiment, however, we found that when IgG from patients and antiphospholipid monoclonal antibodies were compared, IgG from normal controls had no impact on endothelial cells in the expression of procoagulant-anticoagulant molecules such as thrombomodulin, or von Willebrand factor (paper in submission). We believe that the above changes are specific.

Many surface components of endothelial cells, such as thrombomodulin, protein C, and von Willebrand factor, are involved in the balance between anticoagulation and thrombosis [29–33]. Further work must be done to clarify which factors play critical roles after the impact of antibodies on endothelial cells and, if possible, to characterize the different targets among different clones of antibodies. Above all, we conclude that apart from the alteration of blood components after reaction with antibodies, endothelial cells also take part in the initiation of thrombosis when exposed to antibodies.



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# 抗磷脂質抗體激發內皮細胞 — 抗磷脂質症候群導致血管栓塞的一個可能機轉

陳佩佩<sup>1</sup> 林育志<sup>1</sup> 吳昆哲<sup>2</sup> 顏正賢<sup>1</sup> 歐燦騰<sup>1</sup>  
吳正欽<sup>1</sup> 劉宏文<sup>1</sup> 蔡文展<sup>1</sup>

<sup>1</sup>高雄醫學大學附設醫院 過敏免疫風濕內科

<sup>2</sup>吳昆哲婦產小兒科醫院

抗磷脂質抗體症候群為一抗體所誘發的血液高度凝固的疾病，臨床上以動靜脈的重覆栓塞為主要表徵。造成這個疾病的抗體亦是此疾病的特徵，通稱為抗磷脂質抗體。過去的研究對象多集中在此抗體與血中凝固因子的交互作用，然而血管內皮細胞應該有一定的角色；在正常狀況下，當細胞受到刺激時，細胞膜表面的磷脂質會發生反轉現象，而將帶負電的磷脂質轉移到細胞膜外層。我們認為當內皮細胞受到抗磷脂質抗體的作用時，亦會發生此反轉現象，而激發血液的凝固過程。在這個研究裡，我們將從抗磷脂質抗體症候群病人分離出來的 IgG 與人類臍靜脈內皮細胞一起培養後發現人類臍靜脈內皮細胞受到從病人身上所分離出來的 IgG 刺激以後，其細胞表面會有較高的 annexin V 染色，這代表細胞表面有較高之帶負電磷脂質。同時 propidium iodide 的染色並未增加，這代表細胞並未進行細胞凋零。另外，細胞表面的黏著分子 ICAM-1 亦增加。這些發現均代表著內皮細胞在接觸病人的抗體以後出現活化的現象，並可能因此啟發凝血機轉而導致血栓的現象。

**關鍵詞：**抗磷脂質抗體，人類臍靜脈內皮細胞

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通訊作者：蔡文展醫師

高雄醫學大學附設醫院過敏免疫風濕內科

高雄市807三民區自由一路100號