Inhibition of Annexin V Binding to Cardiolipin and Thrombin Generation in an Unselected Population with Venous Thrombosis

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ABSTRACT. ***Objective.*** To examine the effect on annexin V binding to cardiolipin (CL) and *in vitro* thrombin generation by plasma samples from an unselected population of patients with confirmed venous thrombosis and matched controls. The prevalence of autoimmune antiphospholipid antibodies (aPL) was also determined.

Methods. A total of 111 patients who presented to a single emergency room with symptoms suggestive of venous thromboembolic (VTE) disease were studied. In 34 patients the diagnosis of lower limb deep venous thrombosis (DVT) and/or pulmonary embolus (PE) was confirmed (VTE+ group). In the remaining 77 patients the diagnostic workup was negative (VTE– group). Plasma samples were collected prior to the initiation of anticoagulation and examined for IgG anticardiolipin (aCL), IgG anti-ß$_2$-glycoprotein I (GPI), and IgG anti-prothrombin (aPT antibodies) by ELISA. In addition, the effect of individual patient and control plasma samples on annexin V binding to CL and on *in vitro* thrombin generation was determined by a competitive ELISA and a chromogenic assay, respectively.

Results. The prevalence and levels of IgG aCL, anti-ß$_2$-GPI, and aPT antibodies were similar in the VTE+ and VTE– groups. However, plasma samples from the VTE+ group caused a significant inhibition of *in vitro* thrombin generation (mean ± SD Z score: -0.66 ± 0.97 vs 0.26 ± 1.46; $p < 0.001$) and a concurrent but less impressive inhibition of annexin V binding to CL (mean ± SD Z score: –2.53 ± 1.44 vs –2.05 ± 1.61; $p = 0.123$). Upon analyzing a panel of clinical and laboratory variables, only age and inhibition of thrombin generation were significantly associated with VTE disease.

Conclusion. Our findings suggest that subtle abnormalities in annexin V physiology may contribute to the procoagulant state in patients with idiopathic venous thrombosis. (J Rheumatol 2003; 30:1990–3)

Key Indexing Terms:
ANTIPHOSPHOLIPID ANTIBODIES ANNEXIN V THROMBIN

The etiology of venous thrombosis is complex and frequently multifactorial. Antiphospholipid antibodies (aPL) are a recognized risk factor for venous and arterial thrombosis. These are a heterogenous family of autoantibodies that may be detected by antibody binding to immobilized negatively charged phospholipid such as cardiolipin (CL) or by prolongation of an *in vitro* phospholipid dependent coagulation test (lupus anticoagulant, LAC). In patients with the antiphospholipid syndrome (APS), the predominant antibody reactivity is against phospholipid-binding serum proteins, rather than against phospholipid *per se*[1-4]. The most common of these proteins are prothrombin and ß2-glycoprotein I (ß$_2$-GPI), which associate *in vitro* and possibly *in vivo* with negatively charged phospholipids through charge interactions.

The precise pathogenic mechanisms underlying the APS are still unknown. A variety of *in vitro* effects have been attributed to autoimmune aPL including endothelial cell activation[5-7], platelet activation[8-10], and modulation of coagulation mechanisms leading to acquired protein C resistence[11]. Recent studies[12-14] have suggested that inhibition of annexin V binding to negatively charged phospholipids may be an additional pathogenic mechanism. Although autoimmune aPL are causally related to thrombosis in highly selected subsets of patients, their prevalence and pathogenic importance in unselected patient populations presenting with idiopathic thrombosis is less clear. We examined the prevalence and levels of aPL in an unselected population of patients with confirmed venous thrombosis and in matched controls. We also examined the effect of plasma samples on binding of annexin V to CL and on *in vitro* thrombin generation.

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Supported by a grant from the Queen Elizabeth II Health Sciences Centre Research Foundation.

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Submitted November 6, 2002; revision accepted February 13, 2003.

METHODS AND METHODS

Patients. Recruitment of patients and controls to this study occurred through linkage with the Emergency Department Investigation of Thromboembolic Diseases (EDITED) study, which was designed to develop algorithms for the evaluation of patients presenting with suspected deep venous thrombosis (DVT) or pulmonary embolism (PE). Thus, consecutive patients presenting to the Emergency Department of the Queen Elizabeth II Health Sciences Centre, Halifax, Nova Scotia, with clinically suspected DVT (leg pain, swelling, or erythema) or PE (dyspnea, chest pain, hemoptysis, or other cardiorespiratory symptoms) were eligible for this study. Exclusion criteria included: (1) critically ill patients in whom the emergency room physician deemed urgent investigation for VTE was required (e.g., patients with hypotension or severe respiratory failure); (2) patients with suspected axillary or subclavian vein thrombosis; (3) patients whose symptoms had resolved for more than 3 days by the time of presentation; (4) co-morbid conditions making life expectancy less than 3 months; (5) contraindication to contrast media; (6) history of bleeding diathesis or other contraindication to anticoagulants; (7) pregnancy; (8) age less than 18 years; (9) refusal to give informed consent; and (10) geographic inaccessibility to followup. Patients were followed for up to 3 months from the time of presentation. The occurrence of DVT was confirmed by venous ultrasound (US) imaging. The presence of PE was diagnosed by either high probability ventilation-perfusion (VQ) lung scan or low probability VQ scan with either a positive US for DVT or positive pulmonary angiogram. All patients with diagnostic confirmation of DVT or PE were considered to have venous thromboembolic disease (VTE+) and those with a negative diagnostic workup were identified as controls (VTE–). Clinical risk factors for VTE disease were sought and included a past history of DVT or PE, postpartum state, recent immobilization for at least 3 days, the occurrence of major surgery requiring general or regional anesthesia in the preceding 12 weeks, and a strong family history of DVT or PE. Prior to commencing anticoagulation, peripheral blood samples were collected in sodium citrate tubes, centrifuged at 2200 g for 15 minutes and plasma stored at -70˚C until assayed.

ELISA for autoantibodies to CL (aCL). Linbro 96-well EIA microtiter plates (ICN Biomedicals, Costa Mesa, CA, USA) were coated with 45 µg/ml CL (Sigma, St. Louis, MO, USA) in 95% ethanol, 30 µl/well and incubated overnight at 4˚C uncovered in order for ethanol to evaporate. The plates were post-coated with 10% fetal calf serum (FCS, Gibco, Gaithersberg, MD, USA) in 0.02 M Tris buffered saline (TBS), pH 7.4 for 2 h at room temperature. After 3 washes with TBS-Tween, 100 µl of plasma samples diluted with 10% FCS in TBS-Tween (1/100 for IgG) was added to duplicate wells and incubated for 1 h at room temperature. The plates were washed again and 100 µl of alkaline phosphatase conjugated goat anti-human IgG (Sigma) diluted 1/2000 in TBS-Tween containing 10% FCS was added to each well. After incubating at room temperature for 1 h, plates were washed 3 times and color developed in the dark using p-nitrophenyl phosphate (Sigma) in diethanolamine buffer, pH 9.8, 100 µl/well. The reaction was expressed in optical density (OD) units read with a Molecular Devices Emax microplate reader at 405 nm. The results were expressed in GPL units derived from a standard curve using pooled plasma samples from 10 patients with aPL antibodies calibrated against a known standard. A positive result was defined as greater than 2 standard deviations (SD) above the mean of 10 healthy controls.

Purification of $ß_2$-GPI. $ß_2$-GPI was purified from normal human pooled plasma using protein precipitation with 70% perchloric acid followed by high performance liquid chromatography (HPLC) of the supernatant. The purity of the protein was confirmed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using wide molecular weight markers (6.5–205 kDa; Sigma) with both Coomassie brilliant blue and silver staining. The protein concentration was determined by UV spectrometry at 280 nm using the $ß_2$-GPI extinction coefficient of 0.94.

ELISA for anti-$ß_2$-GPI antibodies. Half of the wells in a Corning gamma-irradiated 96-well EIA microtiter plate (Fisher Scientific, Nepean, ON, Canada) were coated with 10 µg/ml of $ß_2$-GPI in carbonate coating buffer, pH 9.6, 50 µl/well. The other half of the plate was coated with carbonate buffer only. Plates were covered and incubated overnight at 4˚C. After 3 washes in 0.02 M TBS, plates were post-coated with 1.2% gelatin in TBS, 100 µl/well for 1 h at room temperature. The plates were washed 3 times in TBS-Tween, and 100 µl of plasma diluted 1/10 with 1.2% gelatin TBS-Tween were incubated in duplicate wells on both the $ß_2$-GPI coated and buffer-coated sides of the plates concurrently for 2.5 h at room temperature. The plates were washed again in TBS-Tween and 100 µl alkaline phosphatase conjugated goat anti-human IgG diluted 1/2000 in TBS-Tween containing 1.2% gelatin was added to each well. After incubating for 1 h at room temperature, plates were washed and the reaction developed in the dark with p-nitrophenyl phosphate in diethanolamine buffer, pH 9.8, 100 µl/well. The OD was read with a Molecular Devices Emax microplate reader at 405 nm. The OD values of the buffer coated wells were subtracted from the OD values of the $ß_2$-GPI coated wells. The results were expressed in arbitrary units using the same pooled standard as for the aCL assay, which was assigned a value of 106 anti-$ß_2$-GPI units/ml. A positive result was defined as greater than 2 SD above the mean of 10 healthy controls.

ELISA for anti-prothrombin antibodies (aPT). This protocol was identical to that for the detection of anti-$ß_2$-GPI antibodies except that the wells were coated with 10 µg/ml prothrombin (Enzyme Research Laboratories, South Bend, IN, USA) in carbonate coating buffer, 50 µl/well. A positive result was defined as greater than 2 SD above the mean of 10 healthy controls.

Chromogenic assay for in vitro thrombin generation. A chromogenic assay was used to determine the effect of plasma samples on the rate of *in vitro* thrombin generation over 20 minutes[15]. Thromboplastin (Sigma) diluted 1/10 in 0.9% NaCl was added to 96-well ELISA plates (25 µl/well) followed by test plasma (25 µl/well). Pooled plasma collected from healthy volunteers was defibrinated by heating to 51˚C for 20 min and centrifugation at 3000 g for 10 min. The supernatant was used as a source of coagulation proteins, added to each well (50 µl/well) and the plate incubated at 37˚C for 10 min. A chromogenic substrate for thrombin (Spectrozyme TH) (American Diagnostica, Greenwich, CT, USA) diluted to 1 mM in 0.9% NaCl (50 µl/well) and 30 mM $CaCl_2$ (50 µl/well) were added sequentially. Background thrombin generation was determined in the absence of thromboplastin. The plates were read immediately and every 2 minutes at 405 nm until thrombin generation had reached a plateau, usually after 20 minutes. Plotting thrombin generation over time yielded a sigmoidal curve. Alteration in the rate of thrombin generation by plasma samples was examined in triplicate wells and the results expressed as a percentage of unaltered thrombin generation (NaCl was added in lieu of sample plasma) using the mean of 3 data points on the linear portion of the curve. The assay was highly reproducible with an intra-plate and inter-plate coefficient of variation of less than 10%. Results of individual samples were expressed in Z values calculated using plasma samples from 10 normal controls. A positive result was defined as a Z score of –2 or less.

Competitive ELISA for annexin V binding to CL. Linbro 96-well EIA microtiter plates (ICN Biomedicals) were coated with 45 µg/ml CL (Sigma) in 95% ethanol, 30 µl/well and incubated overnight at 4˚C uncovered in order for ethanol to evaporate[14]. The plates were post-coated with 100 µl/well of annexin V buffer (20 mM HEPES, 132 mM NaCl, 6 mM KCl, 2.5 mM $CaCl_2 \cdot 2H_2O$, 1 mM $MgSO_4 \cdot 7H_2O$, 5 mM D-glucose, 0.5% bovine serum albumin), pH 7.4 for 1 h at room temperature. After one wash with annexin V buffer, biotin conjugated annexin V (Cedarlane, Fort Washington, PA, USA) in annexin V buffer was diluted 1:1 with plasma samples and added to plates in duplicate wells, 100 µl/well for one h at room temperature. The final concentration of annexin V in the wells was 1 µg/ml. Plates were washed 3 times with annexin V buffer and 100 µl of alkaline phosphatase conjugated Extravidin (Sigma) diluted 1/5000 in annexin V buffer was added to each well. After incubating at room temperature for 1 h, plates were washed 3 times and color developed in the dark using p-nitrophenyl phosphate (Sigma) in diethanolamine buffer, pH 9.8, 100 µl/well. The reaction was expressed in OD units read with a Molecular Devices EMax microplate reader at 405 nm. The results were expressed as

Z values calculated using the OD results from 10 normal controls on each plate. A significant result was defined as a Z score of –2.0 or less (i.e., –2 or more SD below the mean of normal controls), indicating inhibition of annexin V binding to CL by test samples compared to controls. On each plate the binding of annexin V to CL in the absence of patient or control plasma was confirmed.

Statistical analysis. Differences in proportions between groups were examined by chi-square and differences in antibody levels between groups were examined by Student's t test. The association between variables was examined by Pearson correlation coefficient and stepwise logistic regression analysis.

RESULTS

Clinical features of the study and control population. A total of 111 patients were recruited of whom 34 had diagnostic confirmation of VTE disease (DVT only in 23; PE only in 10; DVT and PE in 1). The remaining 77 patients had negative diagnostic investigations and formed the control group (Table 1). The VTE+ group were older (p = 0.003) with a higher proportion of men (p = 0.002). Clinical risk factors for VTE disease were more common in the VTE+ group and reached statistical significance for recent immobilization (p = 0.01) and positive family history of DVT and PE (p = 0.01).

aPL and clinical correlations. The prevalence of aPL in the entire study population was low. A total of 3/111 (2.7%) patients had aCL antibodies, 6/111 (5.4%) had anti-$ß_2$-GPI antibodies, and 16/111 (14.4%) had aPT. There was no difference in the prevalence of autoantibodies between VTE+ patients and controls (Table 2). Similarly, the absolute levels of autoantibodies were comparable in both groups (Table 3).

Inhibition of annexin V binding, thrombin generation, and clinical correlations. Inhibition of annexin V binding to CL, defined as a Z score of ≤ –2, was significantly more common in patients with VTE disease compared to controls [26/34 (77%) vs 39/76 (51%) p = 0.023] (Table 2). Using the same cutoff for thrombin generation (Z score ≤ –2), 2 samples in each patient group [2/33 (6%) vs 2/75 (3%)] caused significant inhibition of thrombin formation (Table 2). Comparison of group means for both of these variables indicated significantly greater inhibition of thrombin generation (p < 0.001) and a concurrent but less impressive difference in the inhibition of annexin V binding to CL (p = 0.123) by plasma samples from patients with VTE disease compared to controls (Table 3). In patients with VTE disease only, there was a weak association between inhibition of annexin V binding and inhibition of thrombin generation (r = 0.33; p = 0.06). Stepwise logistic regression analysis of all clinical and laboratory variables indicated that only age (p = 0.002) and inhibition of thrombin generation (p = 0.006) had a significant association with VTE disease.

Table 1. Clinical features of study (VTE+) and control (VTE–) groups.

	VTE+	VTE–	p
Number	34	77	
Female:male	13:21	55:22	0.002
Age, yrs	60.5 (20–86)	49.2 (21–90)	0.003
Diagnosis			
DVT only	23	0	
PE only	10	0	
DVT and PE	1	0	
Past history of DVT/PE (%)	5 (15)	4 (5)	NS
Postpartum	0	0	NS
Recent immobilization (%)	8 (24)	4 (5)	0.01
Major surgery in last 12 weeks (%)	3 (9)	6 (8)	NS
Strong family history of DVT/PE (%)	4 (12)	0	0.01

Table 2. Proportion of laboratory abnormalities in study (VTE+) and control (VTE–) groups. Elevated IgG aCL, anti-$ß_2$-GPI and anti-PT antibodies were defined as greater than the mean + 2 SD of 10 normal controls. Inhibition of thrombin generation and annexin V binding to CL was defined as less than or equal to a Z score of –2 using 10 normal controls.

	VTE+, n (%)	VTE–, n (%)	p
IgG anti-CL	1/34 (3)	2/77 (3)	NS
IgG anti-$ß_2$-GPI	2/34 (6)	4/77 (5)	NS
IgG anti-PT	4/34 (12)	12/77 (16)	NS
Annexin V binding	26/34 (77)	39/76 (51)	0.023
Thrombin generation	2/33 (6)	2/75 (3)	NS

Table 3. Absolute levels of laboratory abnormalities in study (VTE+) and control (VTE–) groups. IgG aCL expressed as mean ± SD in GPL units. Anti-$ß_2$-GPI and anti-PT expressed in arbitrary units derived from a known standard. Thrombin generation and annexin V binding to CL (mean ± SD) expressed as a Z score derived from 10 normal controls.

	VTE+	VTE–	p
IgG anti-CL (0–11)	2.11 ± 6.13	2.32 ± 5.88	NS
IgG anti-$ß_2$-GPI (0–0.65)	0.13 ± 0.54	0.67 ± 5.31	NS
IgG anti-PT (0–22)	5.8 ± 15.61	8.91 ± 20.38	NS
Annexin V binding	-2.53 ± 1.44	-2.05 ± 1.61	0.123
Thrombin generation	-0.66 ± 0.97	0.26 ± 1.46	< 0.001

DISCUSSION

Antibodies to PL have been implicated in the pathogenesis of both venous and arterial thrombosis in select groups of patients such as those with APS and systemic lupus erythematosus (SLE). We examined the prevalence of aCL, anti-$ß_2$-GPI, and anti-PT antibodies, the major serologic subsets of aPL, in a small but carefully defined unselected population of patients with VTE disease and matched controls. The prevalence and levels of these autoantibodies did not differ significantly between the 2 groups. However, using a functional coagulation assay, there were significant differences in thrombin generation. Plasma samples from patients with VTE disease caused a significant inhibition of thrombin formation compared to controls. There was a concurrent but less impressive inhibition of annexin V binding to CL. Moreover, analyzing a panel of clinical and laboratory vari-

ables, age, and inhibition of thrombin generation were significantly associated with VTE disease.

Previous studies of patients presenting with new onset VTE disease have reported a significant association with the LAC[16,17] but not with the presence of aCL[16,18]. Although we did not measure the LAC in this study, there was an association between the occurrence of VTE disease and inhibition of thrombin generation. We have previously shown that in patients with autoimmune aPL, abnormalities in this functional assay correlate with the presence of the LAC and have a strong association with VTE disease[15]. Our current data suggest that this assay may also detect a subtle procoagulant diathesis in unselected patients presenting with idiopathic VTE disease.

The mechanism responsible for the observed *in vitro* inhibition of thrombin generation and how this phenomenon may promote a procoagulant state is unclear. In the APS it has been proposed that aPL, specifically anti-ß$_2$-GPI, inhibit the binding of annexin V to cell surface negatively charged phospholipids, thereby allowing a permissive procoagulant state. Conversely, *in vitro* binding of aPL to synthetic phospholipid is responsible for the paradoxical inhibition of thrombin generation and LAC activity. In our study, plasma samples from patients with VTE disease caused inhibition of annexin V binding to CL, although the magnitude of this inhibition was markedly less than that seen in patients with autoimmune aPL[14]. Anti-ß$_2$–GPI could not be causally implicated in this phenomenon and thus alternative immunologic or non-immunologic serologic factor(s) may be responsible for this subtle perturbation of coagulation and consequent predisposition to *in vivo* thrombosis.

What are the potential implications of these findings for the management of individual patients with thrombosis? Recent data[19] suggest that annexin-V binding to phospholipid bilayers has sufficient affinity to displace other bound proteins including anti-ß$_2$-GPI-ß$_2$-GPI complexes. In parallel with this observation, Thiagarajan, *et al*[20] found a dose-dependent inhibition of thrombus formation by annexin V in a rabbit carotid artery thrombosis model. They proposed that coating of exposed procoagulant anionic phospholipids on platelets by annexin V was the most likely mechanism of action. Furthermore van Heerde, *et al*[21] showed a reduction in platelet adhesion and fibrin formation on activated endothelial cells in a human *ex vivo* thrombosis model by preincubation with annexin V. These observations raise the possibility that annexin V may be a valuable adjunctive therapy in acute or resistant thrombosis.

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