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Full Length Article

Development of a thrombin generation test in cultured endothelial cells: Evaluation of the prothrombotic effects of antiphospholipid antibodies

Paul Billoir[a], Sébastien Miranda[b], Louise Damian[b], Vincent Richard[c], Ygal Benhamou[b], Véronique Le Cam Duchez[a,*]

[a] Normandie Univ, UNIROUEN, INSERM U1096, Rouen University Hospital, Vascular Hemostasis Unit, F 76000 Rouen, France
[b] Normandie Univ, UNIROUEN, INSERM U1096, Rouen University Hospital, Department of Internal Medicine, Vascular and Thrombosis Unit, F 76000 Rouen, France
[c] Normandie Univ, UNIROUEN, INSERM U1096 EnVI, 76000 Rouen, France

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ABSTRACT

Introduction: Circulating antiphospholipid antibodies (APL) induce vascular injury and endothelial dysfunction, which are associated with thrombotic events and/or fetal loss. We developed a model in which calibrated automated thrombin generation (CAT) is carried out in wells lined with cultured endothelial cells. Then we investigated how far b2GP1 antibodies provoked thrombin generation (TG) enhancing effects in these cells and/or in blood platelets.

Materials and methods: Thrombin generation induced by different concentrations of tissue factor and different levels of endothelial aortic cell confluence was investigated by calibrated automated thrombogram. Endothelial cells were incubated with the purified anti-β2glycoprotein I antibodies of patients with antiphospholipid syndrome (APS). Platelet free plasma and platelet rich plasma were used to study thrombin generation in endothelial cells and platelet reactivity, respectively.

Results: Endothelial cell confluence was negatively correlated with thrombin generation which was dependent on the concentration of APL incubated. Activation of endothelial cells with APL significantly increased thrombin generation triggered by PFP. Triggering by PRP increased thrombinogram parameters. Moreover, anti-β2glycoprotein I antibodies incubated with platelet significantly amplified thrombin formation in PRP and induced platelet activation without tissue factor.

Conclusion: In this *in vitro* study, we demonstrate the feasibility of using thrombin generation test in cultured endothelial cells and suggest the need to realize adjustments to standardize results. The mechanism of prothrombotic states in APS requires endothelial dysfunction and platelet activation. The quantification of thrombin formation shows that APL incubation induces endothelial injury in cultured cells amplified by platelets.

1. Introduction

Antiphospholipid syndrome (APS) is a systemic autoimmune disorder defined by association of vascular injury and persisting antiphospholipid antibodies (APL) [1]. According to several studies and the first results of the RAPS trial, vitamin k antagonist is the first choice drug for thrombotic events in APS [2,3,4]. A recent study evaluated hydroxychloroquine as a promising therapeutic option [5].

The procoagulant effects of APL have been described as overexpression of tissue factor (TF) in patients [6] and in endothelial cells [7]. Moreover, modulation of protein C and protein S expression induces acquired activated protein C resistance (aPCR) [8]. Additionally, a decrease of endothelial nitric oxide synthase [9] and reduced flow-mediated dilatation in APS demonstrating endothelial injury [10] has been observed. Platelet activation in APS and a binding of APL in platelet thrombus have been demonstrated [11]. Activation of the platelet signalling pathway modifies glycoprotein IIb/IIIa conformation and CD 62 expression which induce prothrombotic states [12]. These interactions in the vessel wall predispose to hypercoagulable states. Finding a test to evaluate the recurrent risk of thrombosis in APS patients is essential.

Thrombin generation (TG) induces localized activation of several serine proteases and the final product of the coagulation process is fibrin. Thrombin properties combine pro- and anti-coagulant functions [13]. Thrombin generation test (TGT) evaluates the activation and inhibition of the clotting system [14]. For this reason, TGT has already

* Corresponding author at: Service d'Hématologie Biologique, Centre Hospitalier Universitaire Charles Nicolle, 1 rue de Germont, 76031 Rouen, France.
E-mail address: Veronique.Le-Cam@chu-rouen.fr (V. Le Cam Duchez).

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been used to study APS. APL induce prolonged lagtime and an acquired resistance in presence of activated protein C evaluated with thrombinography [15]. Moreover, in APS, TGT could predict superficial vein thrombosis occurrence [16] and monitor anticoagulation [17]. In parallel, TGT has been developed and validated in cancer cell cultures [18,19,20]. Although TGT is commonly used as an evaluation tool of thrombotic risk, internal quality control is required to standardize results and to reduce intra assay imprecision [21,22,23].

Quiescent endothelial cells (EC) regulate clotting formation, by activating protein C with thrombomodulin and endothelial protein C receptor, and contribute to preventing cell adhesion. Conversely, there is an opposing mechanism of activated EC which facilitate blood coagulation by expressing procoagulant factor and adhesion molecules. Contact phase influence [24] has been reported in EC using TGT, but this approach has not yet been used in APS.

The objective of this study was first, to develop TGT in EC cultures and second, to use TGT in EC cultures and in platelet rich plasma to characterize the direct pathogenic effect of APL.

2. Material and methods

2.1. Endothelial cell cultures

Immortalized endothelial aortic cells TeloHAEC (ATCC® CRL-4052™, United States) were used for TG. Cells were expanded with Endothelial Cell Growth Kit VEGF (ATCC® PCS-100-041™, United States), with 10 units/ml of penicillin and 10 µg/ml of streptomycin (P4333, Sigma Aldrich, France) at 37 °C in 100% humidified atmosphere with 5% CO_2. Adherent cultures were developed in 75 cm^2 culture flasks. At 90% confluence, cells were incubated with trypsin for 5 min at 37 °C then Dulbecco's Phosphate-Buffered Saline was added to neutralise trypsin. Cell suspensions were centrifuged 5 min at 150*g* and pellets were suspended in culture medium. 100 µl of this suspension were spiked in each well in 96-well plates adapted to TG (5000 cells/well). Cells were cultured and adhered at 37 °C in 100% humidified atmosphere with 5% CO_2. Incubation of APL with endothelial cells was realized 4 h before TGT in culture medium.

2.2. Plasma samples

- Platelet free plasma (PFP) samples for TG were obtained from healthy volunteers, who had no history of thrombosis or congenital thrombophilia. Blood samples were taken by antecubital venepuncture and collected in vacutainer tubes containing buffered 0.109 M trisodium citrate (1 part of citrate 3.2%/nine parts of blood). PFP was prepared by double centrifugation of citrate blood for 15 min at 2500*g*.
- Platelet rich plasma (PRP) was prepared by centrifugation of citrated blood for 10 min at 150*g*. Supernatant PRP was removed and adjusted at 150 G/l by autologous PFP obtained after centrifugation of remaining blood for 15 min at 2500*g*. PRP samples were obtained from healthy volunteers without thrombotic or bleeding history and with normal platelet aggregation tests.

2.3. Automated measurement of thrombin generation

In PFP, TG was triggered by a normal concentration of phospholipid (4 µmol/l) and a different concentration of TF (1 or 5 pmol/l) (respectively, low platelet-poor plasma (PPP) reagent or PPP reagent, Diagnostica Stago, Asnières, France). In PRP, TG was triggered by 1 pmol/l of TF (PRP reagent, Diagnostica Stago, Asnières, France) or 5 pmol/l of TF and 1 µmol/l of phospholipid (high PPP reagent with dilution 1:4, Diagnostica Stago, Asnières, France). TG was measured by Calibrated Automated Thrombography and Fluorocan Ascent Fluorometer (Thermoscientific Labsystems, Helsinki, Finland). Experiment was done in duplicate for EC and EC incubated with APL. Four wells were used per group.

2.4. Experimental procedure for thrombin generation test in endothelial cells

Culture medium was removed from the plate. In one well containing EC, 80 µl of PFP or PRP and 20 µl of triggering reagent (mix of TF and phospholipid) were added. In another well, PFP or PRP (Thrombin calibrator, Diagnostica Stago, Asnières, France) was added, then the plate was incubated at 37 °C. A combination of calcium (to begin TG) and fluorogenic substrate (FluCa kit, Diagnostica Stago, Asnières, France) was added. Conversion of fluorogenic substrate was measured and TG parameters (lagtime, endogenous thrombin potential (ETP) and peak concentration) were calculated by Thrombinoscope software (version 3.0.029; Thrombinoscope BV, Maastricht, The Netherlands).

2.5. Isolation of antiphospholipid antibodies

2.5.1. Patients

Blood samples were obtained from six patients with primary arterial APS who fulfilled the international classification criteria for APS [25]. Blood samples were tested according to the updated criteria for lupus-anticoagulant, anti-β2GPI, and anticardiolipin antibodies. Patients were exclusively positive for anti-β2GPI IgG. Written informed consent was obtained from each patient. The study was conducted according to the recommendations set forth by the declaration of Helsinki on biomedical research involving human subjects.

2.5.2. IgG purification

Blood samples were centrifuged for 15 min at 3500*g* to obtain serum and total IgG was purified from serum samples by protein G-Sepharose affinity chromatography (Nab prot G spin column, Fischer Scientific France). Samples from patients with APS or healthy donors were eluted with Tris-glycine (pH 2.5), and then dialyzed against phosphate-buffered saline, concentrated, and solubilized with phosphate-buffered saline (PBS 1X).

The concentration of purified APL IgG was determined using the nephelometric method (BN II system, Siemens Healthineers). The sample containing anti-β2GPI-IgG had an activity of 1860 UA/ml for anti-β2GPI and a concentration of 146.4 UA/ml for anticardiolipin corresponding to 2.48 g/l of IgG. Nonspecific IgG was at 9.48 g/l.

For EC with or without purified APL, TGT was performed in duplicate.

We performed the same protocol for polyvalent immunoglobulin from healthy controls.

2.6. Statistical analysis

All data were expressed as mean ± SD. Data were analyzed with Graphpad Prism 5.0 and with Excel 2016. Mann-Whitney *U* test, Kruskall Wallis test and a Pearson correlation were used. *p*-Values < 0.05 were considered significant.

3. Results

3.1. Effect of endothelial cell confluence on thrombin generation

The addition of PFP and a low concentration of TF (low PPP reagent) did not allow detection of quantifiable TG with calibrated automated thrombogram, even in low confluence (30%).

The addition of PFP and a normal concentration of TF (5 pmol/l) induced quantifiable TG. ETP and peak decreased with confluence (Fig. 1A). Thrombin was not detectable at 100% confluence. We performed a Kruskal Wallis test ($p < 0.001$ for ETP and $p = 0.0086$ for peak) and confirmed a significant difference with a Mann Whitney test (for ETP, $p = 0.047$ between 30% and 50% confluence, and between 50% and 90% of confluence) (for peak, $p = 0.047$ between 30% and