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BRIEF COMMUNICATION

FURTHER INVESTIGATIONS OF LUPUS ANTICOAGULANT INTERFERENCE IN A FUNCTIONAL ASSAY FOR TISSUE FACTOR PATHWAY INHIBITOR

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Tissue factor pathway inhibitor (TFPI) is a natural inhibitor that regulates the initiation of coagulation by inhibiting tissue factor - activated factor VII (TF - FVIIa) in the presence of activated factor X (FXa) (1). Although the precise role of TFPI in haemostasis is yet to be determined, it's ability to inhibit TF - FVIIa in the presence of FXa suggests that is does have an important physiological significance.

TFPI levels have been reported to be normal in a variety of clinical conditions, including patients with lupus anticoagulants (2,3). Lupus anticoagulants (LA) are acquired inhibitors, generally considered to be immunoglobulins, that interfere with *in vitro* phospholipid dependent coagulation tests (4,5). There is current interest in LA due to the associated thrombotic complications as seen in increased risk for venous and arterial thrombosis as well as pregnancy complications (6). We have previously reported decreased functional levels of TFPI in plasma containing LA using a fluid phase assay, but normal levels with a binding assay (7). These low levels of TFPI were shown to be corrected with the addition of excess TF (7).

As a continuation of this study, we again assessed TFPI levels in LA plasma using the same fluid assay. The effect of using three different sources of tissue factor (recombinant, human brain and rabbit brain thromboplastins) in the TFPI assay was examined. Of most significance was the apparent correction of TFPI values as thromboplastin levels increased.

MATERIALS AND METHODS

Reagents and Materials

Purified FVII and FX were purchased from Sigma Chemical Company, St. Louis, USA. A recombinant TF, Innovin® was obtained from Baxter Diagnostics, Deerfield, Illinois, USA. Rabbit brain thromboplastin, Gradiplastin® was kindly donated by Gradipore Ltd, Sydney, Australia. Human brain thromboplastin was extracted and prepared essentially by the method of Hjort (8). The synthetic peptide substrate, S-2222, was purchased from Chromogenix, Molndal, Sweden.

Key Words: Tissue factor pathway inhibitor, tissue factor, lupus anticoagulant.
Abbreviations: TFPI, tissue factor pathway inhibitor; LA, lupus anticoagulant.
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Collection of Samples
Citrated plasma (normal and LA positive) was obtained by drawing 9 parts whole blood into 1 part 0.109 M sodium citrate, and centrifuging at 1200 g at 4°C for 10 minutes. Plasma was collected and then centrifuged again at 3000 g at 4°C for 20 minutes. Venous blood was collected from 20 healthy donors, pooled for use as the reference plasma and stored at -70°C. This plasma was designated as containing 1 U/mL TFPI in the test system used. Plasma (normal and LA positive) was heated at 56°C for 15 minutes, immediately prior to testing, to remove clotting factor activity and fibrinogen. Plasma designated LA positive by accepted criteria (9) (i.e. prolongation of the activated partial thromboplastin time, and increased ratios with Dilute Russell's Viper Venom screening and confirmatory tests), were obtained from clinical laboratories and stored at -20°C until testing.

Fluid Phase Assay
Functional TFPI was measured as previously described (7). Briefly, a reaction mixture was prepared using equal volumes of TF (neat), FVII (0.033 U/mL), FX (0.025 U/mL) and calcium chloride (0.025 M), incubated at room temperature for 30 minutes, then stored at -70°C until required. To the wells of microtitre trays 50 µL of plasma and 50 µL aliquots of reaction mixture were added. After incubation for 10 minutes at 37°C, 50 µL of FX (0.4 U/mL) and S-2222 (2.7 mM) were added and incubated for 25 minutes at 37°C. To terminate the reaction, 50 µL of 50% acetic acid was added and the absorbance read at 405nm on Titertek® Multiskan microtitre tray reader. A five point standard curve was constructed for each concentration of thromboplastin over a range of 0.25 - 2.00 U/mL TFPI using dilutions of normal plasma in imidazole buffer (containing 0.05M imidazole, 0.1M NaCl and 0.0186M HCl).

The TFPI assay was optimised for reagent activity with the three (neat) thromboplastins (recombinant, rabbit brain and human brain) using normal plasma. Neat thromboplastin reagents, as prepared by manufacturer's instructions or published method, each resulted in a prothrombin time of 13 - 14 seconds using normal plasma and also provided a minimum change of 0.3 absorbance units over a range of 0.25 - 2.00 U/mL in the assay system.

Each thromboplastin was tested at various concentrations (x 1/4, x 1/2, x 5, x 10 and x 20) of the neat preparation. Standard curve results were designated acceptable if they met the above criteria. Different concentrations of thromboplastins were prepared by dilution in the recommended diluent or by alternate reconstitution of lyophilised material.

Statistical Analyses
Student's t - test was used to compare the statistical difference between the means of the sets of results. Values of $P < 0.05$ were considered to be statistically significant.

RESULTS

Different concentrations of all thromboplastins were tested for the generation of standard curves. Acceptable results were obtained using recombinant thromboplastin at Neat, x 5, x 10 and x 20 concentrations, human brain thromboplastin at all concentrations tested and rabbit brain thromboplastin at x 1/2 and neat concentrations (Table I). The standard curves for each neat thromboplastin were very similar in their characteristics. It should also be noted that normal plasma as part of each standard curve and a batch of 10 normal plasmas produced normal TFPI results at each tested thromboplastin concentration (results not shown).

TFPI values in 29 LA positive samples were 0.57 U/mL +/- 0.40 U/mL (range: 0.14 - 1.32 U/mL) using neat, 0.66 U/mL +/- 0.30 U/mL (range: 0.21 - 1.42 U/mL) using x 5, 0.79 U/mL +/- 0.36 U/mL (range: 0.32 - 1.90 U/mL) using x 10 and 0.85 U/mL +/- 0.22 U/mL (range: 0.53 - 1.29 U/mL) using x 20 concentrations of the recombinant thromboplastin. There were statistically significant differences between mean TFPI for neat and x 10, neat and x 20, x 5 and x 10, x 5 and x 20 concentrations of recombinant thromboplastin ($P < 0.05$, student's t - test). There were however, no statistically significant differences between the means of the two sets of

results using x 10 and x 20 (P = 0.1434, student's t - test) nor between neat and x 5 concentrations of the recombinant thromboplastin (P = 0.1133, student's t - test).

TFPI values in 29 LA positive samples were 0.39 U/mL +/- 0.20 U/mL (range: 0.20 - 1.12 U/mL) using x 1/4, 0.69 U/mL +/- 0.22 U/mL (range: 0.35 - 1.17 U/mL) using x 1/2, 0.98 U/mL +/- 0.31 U/mL (range: 0.52 - 1.70 U/mL) using neat, 1.11 U/mL +/- 0.44 U/mL (range 0.43 - 1.72 U/mL) using x 5, 1.10 U/mL +/- 0.44 U/mL (range 0.50 - 2.00 U/mL) using x 10 and 1.13 U/mL +/- 0.38 U/mL (range: 0.46 - 1.70 U/mL) using x 20 concentrations of human brain thromboplastin. There were statistically significant differences between x 1/4, x 1/2 and neat concentrations ($P < 0.05$, student's t - test), but not between x 5, x 10 and x 20 concentrations of human brain thromboplastin ($P > 0.05$, student's t - test).

TFPI values in 29 LA positive samples were 0.67 U/mL +/- 0.18 U/mL (range: 0.26 - 1.00 U/mL) using x 1/2 and 1.07 U/mL +/- 0.38 U/mL (range: 0.51 - 1.82 U/mL) using neat rabbit brain thromboplastin. There was a statistically significant difference between the means of the two sets of data (P = 0.0001, student's t - test).

There is a statistically significant difference between the neat mean TFPI using recombinant and neat human thromboplastins (P = 0.0001, student's t - test) and also between neat recombinant and neat rabbit thromboplastins (P = 0.0001, student's t - test). There was no statistically significant difference between the mean TFPI using neat human and neat rabbit thromboplastins (P = 0.1565, student's t - test).

DISCUSSION

Previously, we have shown that TFPI levels in LA positive plasma were significantly reduced (7) when assessed in the fluid phase assay modified from Sandset *et al*, 1991 (10). The modified system used a recombinant thromboplastin as the source of TF in the reaction mixture. In this study, we examined TFPI levels in 29 LA positive samples using three different thromboplastins; recombinant, rabbit brain and human brain.

Essentially similar results were obtained using recombinant thromboplastin as described previously (7) (0.57 U/mL +/- 0.40 U/mL vs 0.36 U/mL +/- 0.19 U/mL). The significantly reduced functional TFPI levels were therefore again demonstrated. The apparent discrepant results when using recombinant thromboplastin require further investigation. We were also able

TABLE I

TFPI Levels of 29 LA Positive Plasmas Assessed Using Three Different Thromboplastins in a Functional TFPI Assay.

Thromboplastin	Recombinant			Human			Rabbit		
Conc.	Mean	SD	Range	Mean	SD	Range	Mean	SD	Range
x 1/4	***			0.39	0.20	0.20 - 1.12	***		
x 1/2	***			0.69	0.22	0.35 - 1.17	0.67	0.18	0.26 - 1.00
Neat	0.57	0.40	0.14 - 1.32	0.98	0.34	0.52 - 1.70	1.07	0.38	0.51 - 1.82
x 5	0.66	0.30	0.21 - 1.42	1.11	0.44	0.43 - 1.98	***		
x 10	0.79	0.36	0.32 - 1.90	1.13	0.38	0.46 - 1.70	***		
x 20	0.85	0.22	0.53 - 1.29	1.10	0.44	0.50 - 2.00	***		

Mean, Standard Deviation (SD) and Range of TFPI values are expressed in U/mL.
*** Results not attainable in TFPI assay system using these concentrations of TF.

to demonstrate the apparent "correction" of these values with the addition of 20 fold recombinant thromboplastin into the assay system, as reported previously (7). As an extension of this study, recombinant thromboplastin concentration at 5 fold and 10 fold quantities in the assay system were tested. Mean functional TFPI was clearly demonstrated to increase as the amount of TF in the system is increased. The increase in TFPI is statistically significant with the addition of x 10 (P = 0.0076, student's t - test) and x 20 (P = 0.001, student's t - test) recombinant thromboplastin.

Two other thromboplastins were also tested in the assay system. Human brain thromboplastin demonstrated normal functional TFPI levels in the assay system (0.98 U/mL +/- 0.31 U/mL). These are very similar results to those reported by Jacobsen *et al*, 1996 (3). Their report does not detail the source of TF, however we assume human brain thromboplastin was used. This study confirmed normal TFPI levels with neat human brain thromboplastin. Further testing was performed with reduced and elevated levels of human brain thromboplastin. When 1/4 and 1/2 fold human brain thromboplastin was used, significantly reduced TFPI results were obtained. Conversely, increased TFPI levels were reported when 5, 10 and 20 fold human brain thromboplastin concentrations were used in the assay system. The differences in these results were not statistically significant. TFPI levels were normal using neat rabbit brain thromboplastin, however this was significantly reduced using 1/2 fold rabbit thromboplastin.

The findings from this study support the hypothesis that TFPI levels determined with the fluid phase assay in LA plasma is dependent on the type and concentration of thromboplastin used. Low TFPI results are again reported using the recombinant thromboplastin unless used at very high concentration. The demonstration of normal TFPI in LA positive plasmas obtained by Jacobsen *et al*, 1996 (3) may be explained with the results obtained in this study using human brain thromboplastin at different concentrations.

TF consists of a specific apoprotein complexed with phospholipids (11). It is probable that LA target the phospholipids of the complex resulting in the interference demonstrated in the TFPI assay. This is supported by the results demonstrated in this study, where TFPI levels in LA plasmas appear to be dependent on decreasing or increasing thromboplastin in the assay system. Crude TF preparations, (e.g. human brain thromboplastin) may provide extraneous membrane/phospholipid as part of the extract milieu as well as the TF complex for LA to target. LA may preferentially target phospholipids not associated with the TF complex resulting in the apparent correction of TFPI values. Studies have commenced to elucidate the effect of additional purified phospholipids in the TFPI assay.

It is clear that the results from this study show that different thromboplastins have varying reactivities in the TFPI assay in the presence of LA, the recombinant thromboplastin in particular showing the greatest sensitivity. This may be due the defined tissue factor apoprotein and phospholipid composition of this reagent. A similar effect has been shown with the use of this recombinant thromboplastin in the dilute prothrombin time assay for LA detection (12).

The results presented in this report clearly indicate the need for adequate levels of TF in the TFPI fluid assay when a LA is present in the test plasma. Excess TF in the reaction mixture, or the use of another technique not affected by LA, for example, the binding assay that has been developed by Berrettini *et al*, 1995 (13) and that modified by this laboratory (7), appeal as alternative techniques and are under further investigation.

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