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Human platelets do not express tissue factor

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ABSTRACT

Background: The controversy about the expression of tissue factor (TF) in platelet after de novo synthesis prevail despite many groups recognize that platelet isolation, assays and reagents, particularly non-specific antibodies, may account for the diversity. In this study the potential of TF expression was evaluated using immune-purified human platelets and employing a very sensitive and highly specific TF activity assay.
Methods: Isolated platelets in plasma anti-coagulated with Fragmin were subjected to stimulation by LPS plus PMA, IgG antibody or TRAP and tested for TF activity.
Results: Platelets stimulated with LPS plus PMA for 4 hours expressed trace amounts of TF like activity (PCA), not inhibited by anti-TF antibody (0.2 ± 0.1 mU/ml blood). Platelets, not immune-adsorbed to remove monocytes, showed significant TF activity (2.0 ± 0.9 mU/ml blood) that was nearly abolished by anti-TF antibody. IgG antibody from patient with lupus anticoagulant failed to enhance the trace amount of PCA as compared to the control in contrast to high TF activity induced in monocytes (0.4 ± 0.1 mU/ml blood versus 27.5 ± 10.5 mU/10^6 cells) showing that activation of complement is not mediating TF expression. Platelet subjected to TRAP activation for 10 min possessed only trace amounts of PCA that was not inhibited by anti-TF antibody and slightly enhanced by anti-TFPI antibody.
Conclusions: It is concluded that platelets free of monocytes do not express TF activity when stimulated by LPS or activated complement factors, implying no role for Toll like receptor (TLR4) as suggested recently. There is no evidence of TF activity associated with platelets as a result of rapid and dynamic process.

Introduction

Platelets play a central role in the hemostatic mechanism by aggregating to form a plug that will stop bleeding, but also as a template for the enzyme complexes essential for thrombin generation.

After the emergence of the concept of blood borne tissue factor (TF) in 1999, an intensive search for the localization of TF in blood cells started [1]. In addition to the expression of TF in circulating monocytes, it was suggested that active TF was also associated with activated platelets [2–5] as well as synthesized by platelets [6,7].

Although we and others have reported that activated platelets do not express TF [8,9], in a very recent response to a study by Bouchard et al. [10], one of the investigating groups still upheld the notion of TF expression in functionally active platelets [11[. The anti-TF antibody (VIC7) used by this group, when tested in our laboratory yielded a positive detection of TF protein on the surface of platelets incubated for 10 min in the presence and absence of Ca-ionophore [12]. In contrast, three other antibody clones proved negative for TF in the same test.

The continuation of reports on TF expression in platelets of healthy subjects as well as patients with various kinds of disease, suggests that independent and high stringency conditions of testing may be required in order to resolve the issue. Thus we subjected activated platelets free of contaminating monocytes to testing in our TF activity assay, the sensitivity and specificity of which exceeds that of flow cytometry. Under these conditions the probability is very low that any significant traces of platelet related TF activity and by implication true TF antigen would escape detection.

Materials and Methods

Affinity purified inhibitory anti-TFPI IgG from goat was generously provided by Novo Nordisk (Copenhagen, Denmark) and polyclonal anti-TF antibody was a kind gift from Dr. L. Vijaya Mohan Rao. Lipopolysaccharide (LPS) (E. coli 026:B6) was obtained from Difco Laboratories, Detroit, Michigan, USA, and Lymphoprep was purchased from Axis-Shield, Oslo, Norway. PMA and TRAP were obtained from Sigma (St Louis, MO).

Abbreviations: FV, Factor V; FVIIa, Factor VIIa; FX/FXa, Factor X/Factor Xa; IgG, immunoglobulin; LPS, lipopolysaccharide; PCA, procoagulant activity; PMA, phorbol myristate acetate; PRP, platelet rich plasma; PS, phosphatidylserine; PSGL-1, P-selectin glycoprotein ligand-1; TF, tissue factor; TFPI, tissue factor pathway inhibitor; TLR4, toll like receptor 4; TRAP, thrombin receptor-activating peptide.

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Blood Collection and Isolation of Cells

Human blood from healthy consenting donors were drawn into a plastic syringe with a 19-gauge needle, and distributed into polycarbonate tubes containing Fragmin (10 IE/ml) (Pharmacia, Uppsala Sweden). The study was approved by the institutional Ethics Committee on human research.

The cell isolation procedures were carried out promptly after collecting the blood. Platelet rich plasma (PRP) was prepared by centrifugation at 150 ×*g* for 15 min. The isolated PRP was spun once more the same way, and then subjected to immune-affinity adsorption of remaining monocytes on MS-columns of microbeads coupled to anti-CD14 antibody (Miltenyi Biotec). The collected monocyte-free PRP was incubated with LPS (100 ng/ml), plus/minus PMA (5 ng/ml) for 4 hrs at 37 °C in a rotary incubator. The platelets were finally isolated by centrifugation at 150 ×*g*, and tested for TF activity. Platelets with remaining traces of monocytes were similarly prepared, but for the omission of the immune-affinity step.

TRAP Activation of Platelets

Platelets used for the experiments with TRAP activation were prepared from 10 ml citrated blood centrifuged at 150 ×*g* to obtain PRP. The isolated PRP was incubated with and without TRAP (10 uM) for 10 min at 37 °C in a rotating incubator. The platelets were then washed twice with 0.15 M NaCl and immediately tested for TF activity, with and without antibodies to TF or TFPI added.

Activation of Whole Blood by LPS Plus PMA or IgG

Whole blood collected in Fragmin was incubated with 5 ng/ml) plus PMA (5 ng/ml) in a shaker incubator at 37 °C at 180 rpm for 4 hours. Mononuclear cells were isolated by centrifugal sedimentation on Lymphoprep as described previously [13] and after washing the cells, they were frozen at -70 °C before testing for TF activity. Isolated and detoxified IgG (88 ug/ml) from a patient with lupus anticoagulant [14,9] was incubated with isolated platelets or whole blood for 3 hours followed by immediate washing of platelets as described above and TF activity measured, whereas mononuclear cells were isolated from whole blood and subjected to TF activity measurements after freezing and thawing the cells.

Quantification of TF Activity

TF was quantified in whole platelets in a two-stage amidolytic assay based on the ability of TF to accelerate the activation of FX by FVIIa, followed by the FXa conversion of prothrombin to thrombin in the presence of activated FV (13). Thrombin was quantified using the Th-1 substrate, and the amount of color generated was determined spectrophotometrically at 405 nm using a microplate reader. Crude rabbit brain extract was used as a standard for TF activity, with an undiluted activity assigned at 1 U/ml. When testing the effect of anti-TF and anti-TFPI antibodies on the TF activity, the antibodies were incubated with the test material for 15 min at 37 °C before testing the TF activity. Since some of the trace amounts of TF activity was not inhibited by anti-TF antibody, PCA has been designed as the TF or TF like activity in the figures.

Results

TF Cannot be Induced in Platelets from which Contaminating Monocytes have been Removed

Isolated, monocyte-free platelets stimulated with LPS plus PMA (a potent Ca^{2+}-ionophor) and vehicle only (control) for 4 hours, expressed a trace amount of TF-like activity (Fig. 1), which was not inhibited by anti-TF antibody. In contrast, a significant amount of TF activity was found in LPS + PMA stimulated platelets that had not been immune-adsorbed, and this activity was nearly abolished by adding an anti-TF antibody (Fig. 1, two columns to the right).

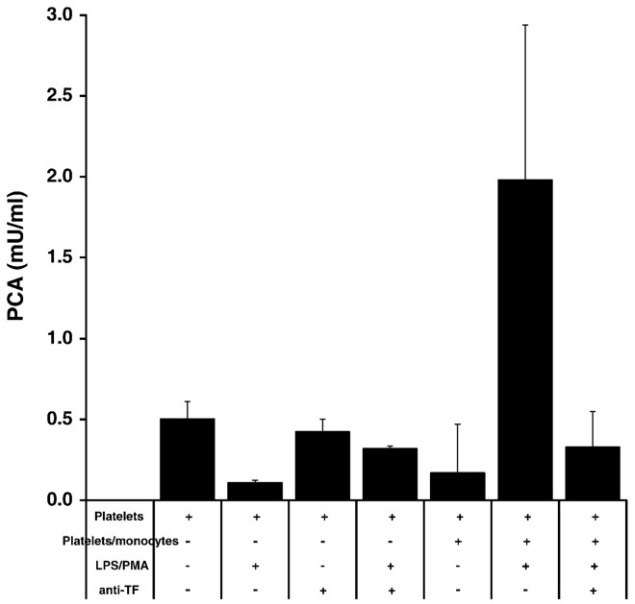

Fig. 1. PCA in platelets, free of monocytes and platelets contaminated with monocytes, stimulated for 4 hours with LPS + PMA. PCA activity measured immediately after isolation. The data are presented as mean, error bars are SD. (n = 5).

Activated Complement System is not Inducing TF Expression in Platelets

In previous work [14] we have seen that IgG from patients with lupus anticoagulant is an extremely potent inducer of TF in monocytes, as also confirmed here When incubating monocyte depleted PRP with IgG, barely any TF activity was found in the platelets after 3 hours whereas high TF activity was induced in monocytes of whole blood. (Fig. 2). The failure of the IgG antibody to provoke the generation of TF activity in platelets, suggests that activation of the complement

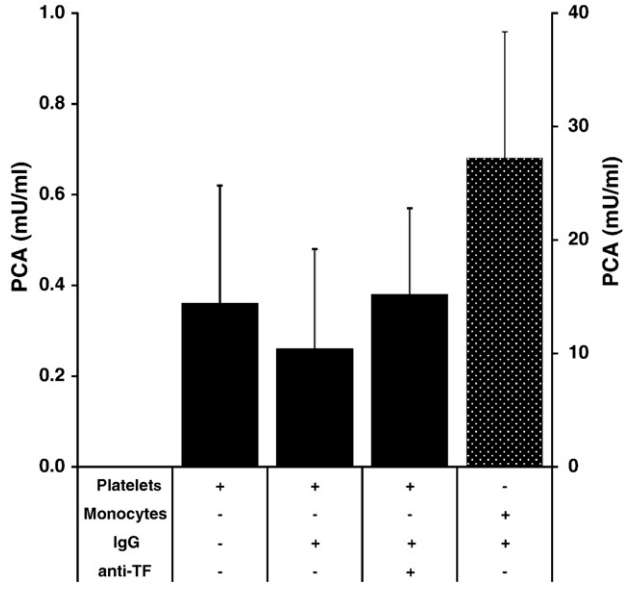

Fig. 2. PCA in platelets free of contaminating monocytes incubated with IgG from patient with lupus anticoagulant for 3 hrs and PCA in monocytes of whole blood stimulated with the same IgG as for platelets. Notice the different Y-axis scale for values in the column to the right. Data are presented as mean, error bars are SD. (n = 3).