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Platelet-related fibrinolysis resistance in patients suffering from PV. Impact of clot retraction and isovolemic erythrocytapheresis

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ABSTRACT

Using patients with polycythemia vera (PV) as an experimental model, we evaluated the impact of clot retraction (CR) and architecture of the clot on fibrinolysis. We studied the kinetics of clot retraction and the fibrinolysis rate in whole blood from 48 PV patients and 48 healthy controls. Measurements were performed before and after isovolemic eryhrocytapheresis (ECP). CR was measured by optical method. Clot lysis time (CLT) and maximum clot firmness (MCF) were measured by thromboelastometry in recalcified blood supplemented with t-PA and tissue factor.

Compared with healthy controls, CR rate in PV patients was higher (0.0219 vs. 0.0138; $p < 0.001$), the clot volume smaller and MCF elevated (64 vs. 58 mm). CR rate correlated with platelet count ($r = 0.546$; $p = 0.001$) but not with erythrocyte concentration ($r = 0.192$; $p > 0.3$). Compared with healthy controls, CLT in PV patients was significantly prolonged (158 min vs. 71 min). Fibrinolysis rate inversely correlated with CR rate ($r = -0.566$; $p < 0.001$); MCF ($r = -0.704$; $p < 0.001$) and platelet count ($r = -0.461$; $p < 0.001$). As judged by confocal microscope, in comparison to healthy controls, clots formed in blood from PV patients demonstrated booth a distinctly higher degree of crosslinking and possessed thinner fibers. Altered CR, MCF and fibrinolysis speeds were not normalized following the ECP procedure. Tirofiban (a blocker of platelet GPIIb/IIIa receptors), unlike aspirin, normalized abnormal CR and fibrinolysis in blood from PV patients. Collectively, our data indicate that in PV patients, abnormal CR may result in formation of thrombi that are more resistant to fibrinolysis. ECP and aspirin failed to normalize platelet-related fibrinolysis resistance.

Introduction

Clot retraction is defined as the slow shrinking of a freshly formed platelet-fibrin clot closely attached to the injured blood vessel wall [1–4]. Retraction of the clot makes possible faster recanalization of the occluded (by thrombus) blood vessel which may result in shortening the time of ischemia of neighboring tissues. The retracted clot is more strongly connected with the vessel wall, more stable mechanically, and thus less prone to detaching under conditions of high shear stress [2].

Clot retraction is also proposed to have great impact on the lysis of a freshly formed platelet-fibrin clot. Studies, carried out on models of fibrinolysis and thrombolysis that properly mimic physiological conditions [2,5–7], strongly indicate that platelets make clots resistant to lysis. This phenomenon has been termed "platelet-mediated fibrinolysis resistance". The mechanism(s) by which human platelets make clots resistant to fibrinolysis are not completely understood, but the accumulated evidence points to clot retraction as one of the major causes. Understanding the cross-talk between clot retraction and fibrinolysis is of great clinical importance since reactive nitrogen species released by activated inflammatory cells have recently been reported to modulate clot retraction rate [8,9].

Although the link between clot retraction and fibrinolysis velocity *in vitro* is well documented [10–12], much less is known about a similar relationship in clinical conditions characterized with altered hemostasis. A prominent example of such a condition is polycythemia vera.

Polycythemia vera (PV) is a chronic myeloproliferative neoplasm, characterized by increased red cell mass and is often accompanied by thrombocytosis and leukocytosis [13–15]. The clinical course of the disease can be complicated by both thrombotic and hemorrhagic events which remain the leading causes of morbidity and mortality in untreated PV patients [15,16]. What's more, both altered clot retraction and fibrinolysis have been reported in PV patients [17,18].

Abbreviations: ASA, aspirin; CR, clot retraction; CLT, clot lysis time; ECP, erythracytapheresis procedure; MCF, maximum clot firmness; PAI-1, plasminogen activator inhibitor-1; PV, polycythemia vera; PRP, platelet rich plasma; RBC, red blood cells; TF, tissue factor; tPA, tissue plasminogen activator; WBC, white blood cells.

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Consequently, this study was undertaken to evaluate the link between clot retraction and fibrinolysis in blood from patients suffering from PV. Another aim was to determine whether normalization of the erythrocyte count by means of routinely performed isovolemic erythrocytapheresis (ECP) affects clot retraction and fibrinolysis in patients suffering from PV.

Materials and Methods

Study Subjects

Forty eight patients (18 men, 30 women) with PV, diagnosed according to criteria established by the World Health Organization [19], and an age-matched control group of 48 healthy volunteers (19 men, 29 women) with normal blood cell counts were entered into the study. Thirty six PV patients took low dose aspirin, whereas 12 of them discontinued aspirin therapy at least one week before blood collection. At the moment of blood collection, patients enrolled in the study did not undergo pharmacological cytoreductive therapy. Healthy volunteers had not taken medication known to affect platelet function and/or coagulation for at least 10 days before blood sampling. None of the study subjects had taken oral contraceptives or hormone replacement therapy for at least two months before blood collection. The study protocol was approved by the Ethics Committee at the Medical University of Bialystok. The procedures were in accordance with the Declaration of Helsinki of 1975, as revised in 2000 and blood samples were obtained with the subjects' informed consent.

Blood Collection and Erythrocytapheresis Procedure

Venous blood was collected with minimum trauma and stasis via a 21-gauge needle (0.8 × 40 mm) into 9 ml polypropylene vacuum tubes (Vacuette, Greiner Bio-One, Kremsmünster, Austria) containing 130 mM trisodium citrate. All PV patients were undergoing erythrocytapheresis (ECP), which was carried out using MCS + cell separator (Haemonetics, Braintree, MA, USA). The removed erythrocyte volume (100-410 ml) was replaced with an equal amount of physiological saline as fluid compensation. The instrument has software which, based on the initial hematocrit, body weight and sex of the subject, precisely determines the blood volume and hematocrit at the end of the procedure. Blood was collected 20 minutes before ECP and an hour after the procedure.

Measurement of Kinetics of Clot Retraction

Measurement of the kinetics of clot retraction in whole blood were performed in non-siliconized glass tubes as described before [8]. Pictures were taken for one hour at 10 min intervals and after 120 min using a digital camera. Quantification of retraction was performed by assessment of the clot area by use of Motic Images Plus 2.0 ML software, and data were processed using Microsoft Excel. Clot surface areas were plotted as a percentage of maximal retraction (*i.e.* volume of platelet suspension). Data were expressed as follows: percentage of retraction (relative clot volume) = (area t_0 − area t)/(area t_0) × 100. Kinetics of clot retraction was characterized by the calculation of rate constant of retraction process.

Thromboelastometric Analyses

Thromboelastometric measurements were performed on the ROTEM® system (Tem International GmbH, Manheim, Germany). Recalcified (10 mM $CaCl_2$) blood was assessed for fibrinolytic potential using either 140 ng/ml tissue factor (TF), and 125 ng/ml of tissue plasminogen activator (tPA). We measured the parameters characterized clot strength (maximal clot firmness) and fibrinolysis (percentage reduction of MCF in time, clot lysis time). The ROTEM analysis was started 30 min after the blood was drawn by adding 320 μl of blood to a cuvette already containing 20 μl of re-calcification reagents.

Evaluation of Platelet-Fibrin Clot Lysis by Fluorimetric Method

Lysis of platelet-fibrin clots was estimated according to Boulaftali *et al.* with some modifications [20]. PRP samples (0.4 ml) were supplemented with Alexa Fluor 488-labeled human fibrinogen (final conc. 75 μg/ml) and incubated at 37 °C for 2 minutes. Afterwards, aliquots of 0.1 ml were transferred to plastic tubes containing 0.775 ml of Tyrode-Hepes buffer supplemented with $CaCl_2$ (final conc. 10 mM) and tissue factor (Innovin, final conc. as indicated), stirred for 30 sec and incubated at 37 °C for 1 hour in the dark to allow full clot retraction. To perform lysis, the retracted clots were transferred to polypropylene tubes containing a 1.5 ml of fresh T-H buffer and incubated at 37 °C for 24 hours in the dark. Then, samples were centrifuged (5 min, 11000 ×*g*, room temperature) to remove clots, and the aliquots (500 μl) of supernatant were collected for determination of fluorescence intensity (Ex 488 nm, Em 522 nm) associated with Alexa Fluor 488-containing fibrin degradation products (AF488-FDP), using the Hitachi F-7000 fluorimeter (Hitachi Ltd., Japan). Fluorescence intensity reflects the progress of fibrinolysis. To determine the maximal fibrinolysis (100% lysis) the retracted clots were incubated 37 °C for 24 hours in the dark with tPA added to the final concentration of 1 μg/ml. In some experiments, PRP samples were incubated with aspirin (final conc. 500 μM) or tirofiban (final conc. 200 μg/ml) for 10 min before the addition of Alexa Fluor 488-fibrinogen.

Confocal Microscopy of Platelet-Fibrin Clots

Samples (100 μl) of PRP were supplemented with Alexa Fluor 488-labeled human fibrinogen (0.15 μM final conc., approximately 15 dye molecules for each fibrinogen molecule) and preincubated for 2 min. at 37 °C. Next, $CaCl_2$ (20 mM final conc.) or $CaCl_2$ + recombinant tissue factor (10 mM and 140 ng/ml final conc. respectively) were added, mixed vigorously and aliquots of 25 μL were transferred to microchamber slides (Ibidi μ-slide VI; Thistle Scientific). Clotting samples were incubated for 2 h at 37 °C in humid atmosphere, protected from light. After gelation time, clots architecture was imaged using fluorescence microscope with confocal imaging system – Nikon ECLIPSE Ti/C1 Plus (Ex 488 nm/Em 515/30 nm, 100× magnification). The images were acquired with a field of view of 120 × 120 μm. At least 10 pictures of different areas of each clot was taken and one representative image is presented.

Routine Hematological Assays

Complete blood cell count, Hct and hemoglobin levels, were measured by an automated hematology analyzer (Sysmex SE9000, Toa Medical Electronics, Kobe, Japan).

Data Analysis

Data reported in this paper are the median of the number of determinations indicated (n). Statistical analysis was performed by Mann-Whitney-Wilcoxon U test and elaboration of experimental data by the use of STATISTICA software (StatSoft, Tulsa, OK, USA). Differences were considered significant at a p value < 0.05. Correlations were assessed by a non-parametric test (Spearman's rank correlation coefficient, r).

Results

Erythrocytapheresis-Evoked Changes in Blood Morphology

The results presented in Table 1 show that the erythrocyte (RBC), leukocyte (WBC) and platelet counts of patients with PV were significantly greater than those of the control subjects. Erythrocytapheresis (ECP) resulted in a significant reduction in erythrocyte count, hematocrit and