Thrombosis Research 133 (2014) 1068–1073

Contents lists available at ScienceDirect

Thrombosis Research

journal homepage: www.elsevier.com/locate/thromres

Regular Article

Platelet microparticle generation assay: A valuable test for immune heparin-induced thrombocytopenia diagnosis

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ARTICLE INFO

Article history:
Received 16 August 2013
Received in revised form 2 December 2013
Accepted 2 December 2013
Available online 9 December 2013

Keywords:
thrombosis
microparticles
heparin-induced thrombocytopenia
platelets

ABSTRACT

Background: Early diagnosis of immune heparin-induced thrombocytopenia (HIT) is essential to improve clinical outcome but remains challenging. The release of platelet microparticles (PMPs) is considered of major pathophysiological significance.
Objectives: The aim of this study was to evaluate performances of PMP generation assay (PMPGA) compared to clinical outcome to diagnose HIT. The second objective was to compare PMPGA with performances of ^{14}C-serotonin release assay (SRA) on the same series of patients.
Methods: Sera of 53 HIT-suspected patients were retrospectively incubated with citrated-whole blood from healthy donors with 1 IU and 500 IU/ml of unfractionated heparin (UH). PMPGA was performed using FACSAria® flow cytometer. The clinical diagnosis was established by two blinded independent investigators analysing in a standardized manner the patient's medical records. Performances of PMPGA and SRA (n = 53) were evaluated using ROC curve analysis with clinical outcome as reference.
Results: In positive HIT patients, PMPs expressing phosphatidylserine are generated with low UH concentration whereas PMP rate decreases significantly in presence of high UH concentration. Using clinical outcome as reference, sensitivity and specificity of PMPGA reached 88.9% (95% CI: 50.7-99.4) and 100.0% (95% CI: 90.0-100.0). Sensitivity and specificity of ^{14}C-SRA were 88.9% (95% CI: 50.7-99.4) and 95.5% (95% CI: 83.3-99.2).
Conclusions: PMPGA is a rapid and reliable assay for HIT diagnosis. PMPGA showed good correlation with ^{14}C-SRA performances and predominately with clinical outcome.

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Introduction

Immune heparin-induced thrombocytopenia (HIT) is a severe immune-mediated adverse effect of heparin treatment that can result in potentially life-threatening conditions such as venous or arterial thrombosis. Venous thrombosis in HIT patients is four-fold more common than arterial thrombosis [1]. HIT consists of an immune response leading to platelet activation, platelet aggregation, production and release of procoagulant platelet microparticles (PMPs), activation of monocytes, endothelial cells and finally to thrombin generation. Platelet activation by pathogenic anti-platelet factor 4 (PF4)-heparin antibodies generates PMPs. Moreover, PMPs serve as a catalytic surface for enhanced thrombin generation, considered as a major component of this reaction [2,3]. These PMPs are characterised by a size ranging from 0.1 µm to 1.0 µm, and by membrane expression of glycoprotein Ib (GPIb, CD42b) and integrin αIIbβ3 (GPIIb-IIIa, CD41/CD61) [4].

Early diagnosis of HIT is essential to improve clinical outcomes. However, this diagnosis remains challenging. The current diagnostic approach consists of the combination of the clinical scoring system ("4Ts score") with immunoassays and functional tests [2,5,6]. Immunoassays [polyspecific antigen assays (IgG/A/M) and the IgG-specific enzyme-immunoassay (EIA)] are acceptable to rule out HIT [2] but are still lacking specificity [7] and need standardization of optical density ranges [8]. Heparin-induced platelet aggregation (HIPA) and ^{14}C-serotonin release assay (^{14}C-SRA) are considered as reference functional assays [9,10]. However, ^{14}C-SRA is time-consuming, technically demanding and requires radioactivity. In addition, this assay is not easily available in routine clinical laboratories and is therefore seldom available to clinicians in real time. Inter-laboratory variability and lack of standardization are also of concern [11,12]. A previous study reported approaches to perform quality control of the SRA [13].

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http://dx.doi.org/10.1016/j.thromres.2013.12.009

The validation of a new gold standard assay would be useful to avoid misdiagnosis [11] and overdiagnosis [14].

We have recently developed a PMP generation assay (PMPGA) in whole blood that could be routinely used for diagnosis of HIT [15]. This assay was not compared with clinical outcome. In the present larger study, we compared PMPGA with SRA and clinical outcome. The ultimate goal is to provide a validated easy to use and rapid functional test with similar or better performances than the standard reference ^{14}C-SRA.

Subjects and Methods

Healthy Subjects

Healthy platelet donors did not take any drug potentially affecting the platelet function for 10 days before the blood sampling.

Patients

After approval by the local ethical committee, 57 patients with suspected diagnosis of HIT at CHU Dinant-Godinne UCL Namur were included in this study. HIT was suspected because of a rapidly decreasing platelet count occurring in hospitalised patients under heparin therapy.

4Ts Score and Clinical Diagnosis

Following HIT suspicion, the "4Ts score" was calculated (based on four criteria: the severity of the thrombocytopenia and its timing, the occurrence of a thrombosis and the exclusion of other causes of thrombocytopenia) [16]. Clinical data were recorded in real time in the hospital medical database. The following information was taken into consideration: patient's medical history, types (fractionated vs. unfractionated) and doses of heparin administered, thrombotic complications, alternative diagnoses, therapeutic attitude, clinical and platelet count evolution, co-suspected medications, and physician's diagnoses [6,17].

Complete compression ultrasonography and multidetector spiral computed tomography were performed for suspected thrombosis.

Patients were classified as positive or negative HIT according to clinical outcome. Clinical outcome were retrospectively and independently confirmed by two investigators (VM and FM), not aware of the results of the laboratory assays. Several clinical criteria have to be fulfilled for the confirmation of clinical HIT diagnosis. Criteria from the ACCP (American College of Chest Physicians) guidelines were used to make the clinical diagnosis of HIT: (i) Thrombocytopenia, defined as at least a 30% decline in the platelet count, with a platelet count increase after heparin cessation; (ii) Timing of platelet count fall after the initiation of heparin occurring between 4 and 14 days, or occurring within 24 to 48 hours (in case of prior heparin exposure within 30 days); and (iii) lack of other, predominant causes of thrombocytopenia [18]. Other causes of thrombocytopenia analysed in this study were: neoplasia, current pregnancy or postpartum, autoimmune disease, sepsis, disseminated intravascular coagulation, intra-aortic balloon pump counterpulsation, multitransfusion, multi-trauma, shock syndrome and drug-induced thrombocytopenia (quinolone, β-lactam, vancomycin, teicoplanin, rifampicin, isoniazid, amphotericin, fluconazole, chemotherapy, anti-GPIIb IIIa; furosemide and proton pump inhibitor). All those 3 clinical criteria have to be fulfilled for the confirmation of clinical HIT diagnosis. Clinical diagnoses made by the 2 local investigators were 100% concordant among them and with conclusions of the medical database.

Blood Sampling and Handling

Briefly, blood was collected with a 20 gauge needle via atraumatic antecubital venipuncture into polyethylene tubes terephtalate Venosafe® (Terumo Europe, Leuven, Belgium) containing buffered sodium citrate (109 mM, nine parts blood to one part sodium citrate solution). A discard tube was used to avoid thromboplastin contamination.

Laboratory Testing

PMPGA and ^{14}C-SRA were performed retrospectively on frozen (−80 °C for maximum 18 months) sera.

Platelet Microparticle Generation Assay (PMPGA)

The PMPGA was performed on the 53 HIT-suspected patients who completed the clinical follow-up.

Briefly, 150 μl of sera of HIT-suspected patients were first incubated 20 minutes at 37 °C with 165 μl of citrated 109 mM whole blood from one appropriate healthy donor (group O Rh + or isogroup ABO and Rh) with 1 IU unfractionated heparin (UH)/ml and 500 IU UH/ml. Platelet microparticles (PMPs) are positive for antiCD41-PE. PMPs negative for annexin-V FITC (phosphatidylserine (PS)) (Fig. 1, Q1) and PMPs positive for annexin-V FITC (Fig. 1, Q2), were quantified on a BDIS FACS Aria® flow cytometer (BD Biosciences, San Jose, CA, USA). The gating strategy involves the following gates: the size of the MPs was defined using a blend of monodisperse fluorescent beads (Megamix, BioCytex, Marseille, France) of three diameters (0.5, 0.9 and 3 μm) according to a previously described protocol [19,20]. The threshold was set on the forward scatter according to ISTH recommendations [19]. In addition, the threshold on side scatter (SSC) was set at the lower limit (i.e. 200 AU). Then, the CD41-Annexin V gate was applied on the MP area for detecting platelet MPs expressing PS (PMPs PS+). After dividing the PE/FITC plot into four quadrants, CD41/PS + MPs would appear in the upper right quadrant. The acquisition started only after one minute to ensure fluidics stability. Flow rate and acquisition time were recorded to calculate the PMP concentration in the samples.

PMP concentrations were measured with 1 IU UH/ml and with 500 IU UH/ml to determine, respectively, PMP concentration generated by HIT antibodies and to check the specificity. Results of PMPGA are expressed as the ratio between PMP annexin V positive (Q2) concentration generated with 1 IU UH/ml and 500 IU UH/ml (rule 1) and as the concentration of PMPs annexin V positive (Q2) generated at 1 IU UH/ml (rule 2).

The flow rate was determined by recording during 10 minutes a known number of beads included in a TruCount® tube (BD biosciences). This tube contains a mix of serum and whole blood of a healthy subject (in proportions mentioned above). The aim was to have a similar viscosity index that in the test sample. The measurement was performed each 60 sec until 10 minutes with a coefficient of variation lower than 10%.

^{14}C-Serotonin Release Assay

The ^{14}C-serotonin release assay was carried out according to previously published protocols on the 53 HIT-suspected patients who completed the clinical follow-up [6,10].

Data Analysis

Statistical analysis was performed using Medcalc software (version 10–4.8) (Gent, Belgium).

ROC Curves were performed to determine the optimal cut-offs of PMPGA for rule 1 and rule 2 compared to clinical outcome. When indicated, comparison of ROC Curves was also performed.

Area under the curve, sensitivity and specificity of PMPGA (rule 1, rule 2, rule 1 + 2), ^{14}C-SRA and their 95% CI were calculated with clinical outcome as reference.

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