Thrombosis Research 136 (2015) 1291–1298

Contents lists available at ScienceDirect

Thrombosis Research

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

Full Length Article

Thrombin generation and international normalized ratio in inherited thrombophilia patients receiving thromboprophylactic therapy

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ARTICLE INFO

Article history:
Received 29 July 2015
Received in revised form 30 September 2015
Accepted 13 October 2015
Available online 19 October 2015

Keywords:
Endogenous thrombin potential
International normalized ratio
Thrombin generation
Thrombophilia
Thromboprophylactic therapy
Thrombotic and hemorrhagic risk

ABSTRACT

Background: Thrombin generation assay (TGA) is useful as a global functional test for assessing bleeding or thrombotic risk and its modification with therapy. We investigated TGA to assess anticoagulation status compared with the international normalized ratio (INR) system in patients with primary thrombophilia receiving and not undergoing thromboprophylaxis.
Materials and methods: We studied 50 patients with at least one thrombotic event and a confirmed diagnosis of inherited thrombophilia. Thrombin generation was measured in platelet-poor plasma by calibrated automated thrombography (CAT).
Results: Patients in optimal anticoagulation (INR: 2.0–3.0) showed an endogenous thrombin potential (ETP) of 14–56% of normal and a peak of 18–55% of normal. A significant inverse relationship between INR and thrombin generation parameters (ETP, peak and velocity index) and a linear correlation for lag time was found in patients treated with vitamin-K antagonists (VKA). Receiver-operating characteristics (ROC) analysis showed that the optimal cutoff for ETP was 1600.2 nM·min (111.6% of normal, with a sensitivity of 96.6% and a specificity of 92.9%) and for the peak was 298.3 nM (112.1% of normal, with a sensitivity of 96.4% and a specificity of 100%). According to this analysis, ETP was able to identify patients with increased thrombotic and hemorrhagic risk, correlating with severe clinical complications.
Conclusion: TGA showed excellent sensitivity and specificity for assessing anticoagulation status in patients with primary thrombophilia receiving VKA, with significant advantages with regard to INR. Clinical data strongly support ETP as a valuable indicator of thrombotic or hemorrhagic risk in patients receiving or not receiving thromboprophylaxis.

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1. Introduction

Thrombosis is a major cause of morbidity and mortality in the Western world and is considered to be the third leading cause of death in the general population [1]. A disturbance of hemostasis, the so-called thrombophilia state featuring hypercoagulability or hypofibrinolysis, is central in the pathogenesis of either venous or arterial thrombosis [2]. Venous thromboembolism (VTE), a chronic, multifactorial, and potentially fatal disease, accounts for 75% of all thromboses [3] and occurs when two or more risk factors are present at the same time [4]. There are, however, patients in whom VTE is considered unprovoked or idiopathic [5].

The development of novel and sophisticated laboratory techniques has made possible the identification of unknown genetic and acquired risk factors for VTE. Mutations in factor V (FV Leiden) and factor II (FIIG20210A) genes have been identified as the most common inherited risk factors [6,7]. These VTE susceptibility mutations are present in 5–10% of the general population and in at least 40% of patients with VTE [8]. Deficiencies of natural coagulation inhibitors such as antithrombin, protein C (PC), and protein S (PS), high clotting factor

Abbreviations: aPTT, Activated partial thromboplastin time; α^2M-Thrombin, Alpha^2 macroglobulin–thrombin; Anti-FXa, Anti-factor X activated; CAT, Calibrated automated thrombography; DVT, Deep venous thromboembolism; ETP, Endogenous thrombin potential; FVIII, Factor VIII; FVIII:C, Factor VIII coagulant activity; FVL, Factor V Leiden, FV Leiden; FIIG20210A, Factor II G20210A; INR, International normalized ratio; LMWH, Low-molecular-weight heparin; PC, Protein C; PE, Pulmonary embolism; PLT, Platelet therapy; pM, Picomolar; PNP, Pooled normal plasma; PPL, Phospholipids; PPP, Platelet-poor plasma; PRP, Platelet-rich plasma; PS, Protein S; PT, Prothrombin time; ROC, Receiver-operating characteristics; TF, Tissue factor; TGA, Thrombin generation assay; TG, Thrombin generation; VKA, Vitamin-K antagonists; VTE, Venous thromboembolism.

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http://dx.doi.org/10.1016/j.thromres.2015.10.026
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levels (factors VIII, IX or XI), hyperhomocysteinemia, and high levels of phospholipid antibodies are also associated with an increased risk of VTE [5]. High-risk patients have deficiencies of natural anticoagulants and are homozygous for FV Leiden and FIIG20210A mutations; heterozygous individuals exhibit a moderate risk but a comparable increased risk is provided if they also have multiple VTE-associated abnormalities [8].

Different approaches such as the Vienna model or thrombin generation (TG) have attempted to predict VTE recurrence or to measure thrombosis risk by means of assessing physiological or secondary conditions of hypercoagulability [9,10], even in healthy individuals [11,12]. These studies pursue objective criteria to measure a prothrombotic phenotype and establish the duration of antithrombotic therapy according to the individual genetic and environmental risk. It is well recognized that patients with VTE provoked by surgery, trauma, immobilization, pregnancy or female hormone intake are at low risk of recurrence [10].

Thrombin generation is the result of prothrombin conversion and thrombin inactivation. It is known that reduced TG is found in patients with bleeding tendencies such as patients with hemophilia [13]. In contrast, increased TG is associated with a prothrombotic phenotype and high risk for VTE [9,14]. Increased TG can be caused by an excess of prothrombin, overactive prothrombin conversion (FVL and deficiency of PC or PS) or by decreased thrombin breakdown (antithrombin deficiency) [15].

Because VTE is a multicausal disease, a global laboratory test that reflects the multiple factors underlying the activation of the hemostatic system would be useful for predicting recurrence risk in thrombosis. TG assay (TGA) has been used as a global functional test to evaluate hemostatic capacity in patients with various disorders as well as to assess bleeding or thrombotic risk and its modification with antithrombotic or hemostatic drugs [16,17]. Because all possible causes of thrombophilia increase TG and antithrombotic therapy decreases the formation of thrombin [18], one might expect that TG measurements could be useful for monitoring anticoagulant therapies [11]. Therefore, TGA could help to determine the overall risk of VTE recurrence [9]. This study aims to investigate the usefulness of TGA in patients with primary thrombophilia to assess the anticoagulation status compared with the international normalized ratio (INR) system in a subgroup of patients receiving oral anticoagulants and to evaluate the behavior of TGA in patients not receiving thromboprophylaxis therapy.

2. Materials and methods

2.1. Subjects

Fifty patients who experienced at least one thrombotic event were selected after a diagnosis of inherited thrombophilia was confirmed via heterozygosity for FVL or FIIG20210A or by a deficiency of antithrombin, PC or PS. Twenty-nine patients were being treated with vitamin-K antagonists (VKA) (warfarin or acenocumarol), one patient received low-molecular-weight heparin (LMWH), and five patients received antiplatelet therapy (aspirin or clopidogrel); the remaining 15 patients had discontinued therapy at least 1 month prior to their enrollment in the study. We obtained data on clinical features, familial and personal thrombotic history, acquired risk factors, thrombotic event(s), and treatment and evolution of the last thrombotic episode from each patient. Arterial or venous thromboses were confirmed according to accepted criteria [19]. The study was approved by our local research and ethics committee and written consent was obtained from all participants.

2.2. Sample collection and plasma preparation

We collected peripheral venous blood in Vacutainer polypropylene tubes (Becton Dickinson, Franklin Lakes, NJ, USA) containing 109 mmol L^{-1} trisodium citrate (9:1 v/v). Platelet-poor plasma (PPP) for TGA was obtained by double centrifugation at 3000 g for 15 min at room temperature and collected from the upper half volume of the plasma supernatant, aliquoted into cryovials and frozen at −80 °C until use. For routine coagulation tests, PPP was obtained from the remaining plasma and aliquoted for immediate processing or frozen at −80 °C. PPP was always prepared within 30 min after venipuncture. For blood grouping, 2 mL of whole blood in a dry tube without anticoagulant was also obtained.

Pooled normal plasma (PNP) was prepared by mixing PPP from 20 healthy volunteer blood donors (16 men and 4 women) between 21 and 58 years of age (mean age: 41.2 ± 11.1 years). Subjects had no history of VTE or bleeding disorders or were using drugs known to affect the coagulation system.

2.3. Routine laboratory and thrombophilia tests

Activated partial thromboplastin time (aPTT), prothrombin time (PT), PT–INR, and ABO blood grouping were determined according to routine laboratory methods. Thrombophilia was previously investigated. PC activity was measured via a coagulometric method with Protein C Reagent (Dade Behring, Siemens, Anaheim, CA, USA). Total and free PS antigens were measured using coagulometric Protein S Ac Reagent (Dade Behring). AT activity was measured via a chromogenic assay with a Berichrom Antithrombin III (A) Kit (Dade Behring). Factor VIII was measured via a one-stage clotting assay using factor-deficient plasma (Dade Behring). All measurements were performed in a BCS XP system (Dade Behring) according to the manufacturer's instructions. We considered a clotting abnormality if the FVIII activity, PC activity, free PS antigen or AT activity values were not within the reference range. Hereditary deficiency of AT, PC or PS was confirmed in a second plasma sample 2–3 months after initial testing. PC and PS values were established in the absence of VKA administration. FVL and FIIG20210A mutations were detected using a multiplex polymerase chain reaction protocol [20].

2.4. Thrombin generation measurements

We measured TG using the calibrated automated thrombography (CAT) method according to Hemker et al. [21] with some modifications. We used a 96-well plate Fluoroskan Ascent fluorometer (ThermoLab Systems OY, Helsinki, Finland) equipped with a dispenser and a 390/460 nM filter set (excitation/emission) to detect fluorescence intensity. All TG reagents (PPP-Reagent, α^2 M-Thrombin Calibrator with 600 nM human thrombin, FluCa kit: Fluo-buffer and Fluo-substrate Z-Gly-Gly-Arg-amino-methyl-coumarin) were purchased from Stago (Diagnostica Stago, Inc., Parsipanny, NJ, USA), and the 96-well round-bottomed microtiter plates were from Immulon 2-HB (ThermoLab Systems, Woburn, MA, USA). Human Thrombomodulin (TM) was purchased from PeProtech (PeProtech-Mexico, Mexico City); TM was reconstituted as recommended by the manufacturer and used immediately or stored at −80 °C. Briefly, 80 μL of PPP was dispensed into the wells of the microtiter plate. We next added 20 μL of PPP-Reagent-containing tissue factor (TF) and negatively charged phospholipids (PPL) to obtain a final concentration of TF 5.0 pM and PPL 4 μM. TM was only added to PPP of non-anticoagulated patients or patients receiving platelet therapy at a final concentration of 9 nM. The starting reagent (20 μL per well) contained the fluorogenic substrate and $CaCl_2$. All experiments were performed in duplicate (specified exceptions), and the microplate was placed in the fluorometer at 37 °C for 10 min before the measurement. A dedicated software program, Thrombinoscope v.5.0.0.742 (THROMO-AB-30, Thrombinoscope BV, Maastricht, The Netherlands), allowed us to calculate thrombin activity. The most important TG parameters calculated included lag time, peak thrombin, endogenous thrombin potential (ETP) corresponding to the area under the curve, and velocity index. Normal TGA parameters were established as the average value

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