Thrombosis Research 131 (2013) 457–462

Contents lists available at SciVerse ScienceDirect

Thrombosis Research

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

Regular Article

Detection of hypofibrinolysis in stable coronary artery disease using the overall haemostatic potential assay

Caroline J. Reddel [a], Jennifer L. Curnow [a,b], Jasmin Voitl [a], Alexander Rosenov [a], Gabrielle J. Pennings [a], Marie-Christine Morel-Kopp [c,d], David B. Brieger [a,e,*]

[a] ANZAC Research Institute, University of Sydney, Australia
[b] Department of Haematology, Concord Repatriation General Hospital, Australia
[c] Northern Blood Research Centre, Kolling Institute, University of Sydney, Australia
[d] Department of Haematology and Transfusion Medicine, Royal North Shore Hospital, Australia
[e] Department of Cardiology, Concord Repatriation General Hospital, Australia

ARTICLE INFO

Article history:
Received 21 November 2012
Received in revised form 28 February 2013
Accepted 19 March 2013
Available online 11 April 2013

Keywords:
Coronary artery disease
fibrinolysis
overall haemostatic potential
thrombin generation

ABSTRACT

Introduction: Patients with stable coronary artery disease (CAD) are at risk of arterial thrombosis causing myocardial infarction. Detection of global haemostatic markers of hypercoagulability and hypofibrinolysis may be important for risk stratification and individualised treatment. We examined overall haemostatic potential (OHP) and thrombin generation in a group of stable CAD patients. We also sought to investigate associations between fibrinolytic inhibitors and abnormal global fibrinolysis in these patients.
Materials and Methods: Blood samples were collected from 56 patients defined by coronary anatomy as symptomatically stable CAD. Medications were recorded. Samples were analysed using the global coagulation assays OHP and thrombin generation (calibrated automated thrombogram, CAT), platelet aggregometry measured by Multiplate®, and levels of plasminogen activator inhibitor-1 (PAI-1) antigen measured by ELISA. Results were compared with a reference group of healthy controls.
Results: Stable CAD patients displayed increased fibrin and thrombin generation and impaired fibrinolysis (decreased overall fibrinolytic potential, OFP, and increased clot lysis time) compared with healthy controls. No effect of antiplatelet agents or other medications on these parameters was observed using platelet-poor plasma. After multivariate adjustment, OFP of healthy individuals was significantly associated with fibrinogen, but in CAD patients PAI-1 became an important determinant.
Conclusions: Hypercoagulability of plasma is observed in stable CAD, with both increased thrombin generation and reduced fibrinolytic potential making a significant contribution. The OHP assay may provide a simple method of identifying hypercoagulability in individual patients.

Introduction

Hypercoagulability, an increased tendency of the blood to form pathological clots, represents an imbalance in the haemostatic system. Stable coronary artery disease (CAD) is predominantly caused by coronary atherosclerosis, an inflammatory disease which predisposes the individual to arterial thrombosis, and most myocardial infarctions (MI) are caused by thrombus formation on the plaque surface [1]. The risk of MI in patients with stable coronary disease is attenuated by modification of hypercoagulability-related risk factors such as smoking, obesity and hypercholesterolemia [2]. In addition, there is evidence for a link between markers of fibrinolysis and the prognosis of stable and acute cardiovascular disease [3–6].

Antiplatelet and anticoagulant treatments are effective methods of reducing myocardial infarction in high risk groups, but there is an associated risk of bleeding which mitigates against the long-term use of combined antiplatelet and anticoagulant therapies [7–10]. Therefore, detection of haemostatic markers that may predispose to an increased risk of coronary thrombosis is likely to be important for risk stratification and to design individual approaches to treatment.

The haemostatic system reflects the complex interplay between coagulation factors and inhibitors. Thrombophilias may be identified by detecting abnormal levels of factors, but individual test results may not be clinically useful when patients are at increased risk of thrombosis due to a combination of risk factors. Therefore, global assays of coagulation are becoming widely recognised as more

Abbreviations: ACS, acute coronary syndrome; CAD, coronary artery disease; CAT, Calibrated Automated Thrombogram; CLT, clot lysis time; CRP, C-reactive protein; MI, myocardial infarction; OCP, overall coagulation potential; OD, optical density; OHP, overall haemostatic potential; OFP, overall fibrinolytic potential; PAI-1, plasminogen activator inhibitor-1; rtPA, recombinant tissue plasminogen activator; TAFI, thrombin activatable fibrinolysis inhibitor.

* Corresponding author at: Department of Cardiology, Concord Repatriation General Hospital, Hospital Rd, Concord, NSW, 2139, Australia. Tel.: +61 297677282; fax: 61 297676994.
E-mail address: davidb@email.cs.nsw.gov.au (D.B. Brieger).

0049-3848/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.thromres.2013.03.015

informative in the detection of the overall functional balance of the haemostatic system in an individual [11,12]. Previous work using the Calibrated Automated Thrombogram (CAT), a global haemostatic assay measuring *ex vivo* thrombin generation potential, showed high thrombin generation in acute coronary syndrome (ACS) patients compared with stable CAD patients, but also implied elevated thrombin generation in stable CAD patients compared with controls [13].

Considering the importance of impaired fibrinolysis, global assays should also include measures of fibrinolytic activity. Single fibrinolysis parameters, while suggesting a link between impaired fibrinolytic activity and severity or recurrence of CAD, have not been shown to be good predictors of acute coronary events, but overall fibrinolytic function may have better predictive value [5]. Indeed, identification of the combination of factors contributing to increased thrombus formation and decreased fibrinolysis may be even more powerful.

The overall haemostatic potential (OHP) assay is a simple and cost-effective method for observing *ex vivo* fibrin generation and fibrinolysis over time [14]. Studies using this assay showed a significant reduction in fibrinolytic potential (and to a lesser extent, increase in fibrin generation) in ACS patients more than three months after first MI [15]. However little is known about the effect of underlying stable CAD, without recent MI, on plasma potential for fibrin generation and fibrinolysis.

We sought to validate the OHP assay as a method of measuring hypercoagulability in a group of stable CAD patients, by comparing the results with the CAT assay, and also to examine underlying fibrinolysis in this patient population.

Materials and Methods

Subjects

Ethical approval was obtained from the hospital ethics committee (ref #CH62/6/2009-91). Subjects were consecutive patients with stable angina pectoris presenting at the cardiac catheterisation laboratory for either percutaneous coronary intervention or diagnostic angiography. All had significant CAD upon conventional coronary angiography. Patients who had taken heparin or low molecular weight heparin in the last 24 h and patients on warfarin were excluded. Samples were collected before the procedure.

The reference groups were made up of healthy volunteer blood donors recruited either from the Australian Red Cross Blood Service (ARCBS), Clarence St Centre, Sydney, NSW, or Concord Repatriation General Hospital. Each donor gave written informed consent. In all groups, donors had no acute illnesses, no vascular disease and had not taken anticoagulants within the seven days prior to donation. Age, gender and current medications were recorded.

Sample Collection and Processing

For OHP, CAT, and ELISAs, blood samples were collected from the cubital vein with a 21 gauge butterfly needle. The tourniquet was removed and the first 3 ml of blood discarded. Blood was collected into BD Vacutainer 0.109 M sodium citrate tubes and hirudinised tubes (Becton-Dickinson, Franklin Lakes, NJ, USA) for platelet aggregometry. Citrated blood was then centrifuged for 10 min at 20 °C and 2500 ×*g*. The plasma supernatant was centrifuged under the same conditions for a further 10 min to produce platelet-poor plasma (PPP), which was stored at −80 °C. Thrombin activatable fibrinolysis inhibitor (Asserachrom TAFI, Diagnostica Stago, Gennevilliers, France) and plasminogen activator inhibitor-1 (TriniLIZE PAI-1 Antigen, Trinity Biotech, Bray, Ireland) antigen levels were measured by ELISA.

The standard clotting assays prothrombin time, International Normalised Ratio (INR), activated partial thromboplastin time and Clauss fibrinogen were performed on an automated coagulation analyser, the STA-R (Diagnostica Stago). Reagents were from Dade-Behring Diagnostics, (N.S.W., Australia) and Diagnostica Stago. Platelet count was obtained by optical and impedance methods on Abbott Diagnostics CELL-DYN Sapphire. C-reactive protein (CRP) concentration was measured by the Dimension® particle enhanced turbidometric immunoassay on Siemens Dimension RxL Max Analyzer.

Platelet Aggregometry

The effect of antiplatelet therapy was tested in duplicate by whole blood impedance using the Multiplate® (multiple electrode platelet aggregometry) analyser (Verum Diagnostica GmbH, Munich, Germany). This method measures activated platelet binding to two sets of electrodes through change in conductivity. Aggregation was stimulated with arachidonic acid (ASPI test), adenosine diphosphate (ADP test) and thrombin receptor activating peptide-6 (TRAP test). Changes in impedance were recorded over a 6 minute period. Results were expressed as area under the curve (AUC) using arbitrary units.

OHP Assay

A modified tissue factor triggered OHP assay based on that previously described [16] was used. Fibrin time curves were generated in microtiter plate wells at 37 °C and PPPs were tested in duplicate. Overall coagulation potential (OCP) curves were generated in microtiter wells containing 75 μL PPP and 75 μL OHP buffer (final concentrations in the well: Tris 33 mmol/l, NaCl 65 mmol/l, $CaCl_2$ 16.5 mmol/l (pH 7.0) and tissue factor 0.85 pmol/l). Automated absorption measurements were taken at 405 nm every minute for 60 min. OHP curves were generated using a similar method, except the added buffer also contained recombinant tissue plasminogen activator (rtPA) to give a final concentration of 300 ng ml^{-1}. Values for OCP and OHP represent the area under the relevant fibrin time curve calculated by summation of absorption values [14,17]. The overall fibrinolysis potential (OFP) value was calculated by (OCP-OHP)/OCP × 100% and represented the area under the fibrinolytic portion of the curve as a percentage of the total OCP value. Max OD was the mean of the maximum optical density (OD) reached in the duplicate OCP curves. Slope was calculated progressively for each OD reading on the OCP curve, using three time points, and the greatest increase in OD for these points represented the Max slope, as previously described [18]. Delay to fibrin clot formation was calculated using the intercept of the Max slope. Clot lysis time (CLT) was defined as the time between the mid-point of the clear-to-turbid transition and the turbid-to-clear transition.

Calibrated Automated Thrombogram

Thrombin generation was measured according to the method described by Hemker et al. [19] in a Fluoroscan Ascent fluorometer (Thermo Labsystems, Helsinki, Finland). Briefly, 80 μl of PPP were dispensed into the wells of Immulon microplates 2HB from Thermo Labsystems (Helsinki, Finland); 20 μl of calibrator (Diagnostica Stago) or PPP reagent (9 pM rTF and 24 μM procoagulant phospholipids in HBS buffer pH 7.35 (20 mM Hepes, 140 mM NaCl and 0.5% BSA)) were added per well. Thrombin generation was triggered by the automatic dispensing of 20 μl of a solution containing 0.1 M CaCl2 and 2.5 mM fluorescent substrate (Z-Gly-Gly-Arg-AMC, Calbiochem, San Diego, CA, USA). Fluorescence was measured every 20 seconds over a 60 minute period. All tests were performed in triplicate. The Thrombinoscope software program (Thrombinoscope BV, Maastricht, The Netherlands) was used to calculate the endogenous thrombin potential (ETP) which is the main endpoint of total thrombin generation potential together with the peak thrombin.