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Regular Article

Real-time measurement of free thrombin: Evaluation of the usability of a new thrombin assay for coagulation monitoring during extracorporeal circulation

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ABSTRACT

Introduction: In patients undergoing cardiac surgery with heart-lung machine support, adequate anticoagulation to mitigate blood clotting caused by the artificial surfaces of the extracorporeal circulation (ECC) system is essential. These patients routinely receive heparin, whose effectiveness is monitored by measurements of the activated clotting time (ACT). However, ACT values only poorly correlate with the actual hemostatic status. The aim of our study was to evaluate the detection of free thrombin in heparinized human blood as a monitor of anticoagulation during ECC.

Materials and Methods: Human whole blood was anticoagulated with different concentrations of heparin (0.75, 1, 2 or 3 IU/ml) and circulated in the Chandler-loop model for up to 240 min at 37 °C. Next to ACT, ECC-mediated changes in free active thrombin, prothrombin fragment 1 + 2 (F1 + 2) and thrombin-antithrombin-III (TAT) levels were measured before and during circulation. Platelet activation and cell count parameters were further investigated.

Results: Our study shows that detection of ECC-mediated changes in free thrombin is possible in blood anticoagulated with 0.75 or 1 IU/ml heparin, whereas no thrombin was detectable at higher heparin concentrations. Thrombin generation during 240 min of ECC is comparable to F 1 + 2 and TAT plasma levels during ECC.

Conclusions: Thrombin is the key enzyme in the coagulation cascade and hence represents a promising marker for monitoring the coagulation status of patients. Although detection of free thrombin was not feasible at high heparin concentrations, the employed test represents an additional test to current laboratory methods investigating blood coagulation at low heparin concentrations.

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Introduction

Already in 1954, extracorporeal circulation (ECC) with heart-lung machine (HLM) support was firstly applied to a human and is nowadays routinely used during cardiopulmonary bypass (CPB) surgery to maintain stable circulatory parameters of the patient and to guarantee gas exchange [1]. ECC is further employed in several other procedures as extracorporeal membrane oxygenation (ECMO), extracorporeal life support (ECLS), dialysis and for ventricular assist devices (VAD). Nevertheless, blood contact with the artificial surfaces of the ECC systems as well as shear forces induce activation of the hemostatic system resulting in hemostatic and thrombotic complications, which represent further life-threatening factors for already critically ill patients [2–4]. Until today, the effect of ECC on the hemostatic system and the respective anticoagulation management are still important research topics.

The human hemostatic system is physiologically regulated in a complex manner to warrant save arrest of bleeding as well as to prevent coagulopathy. Hemostasis comprises three steps defined as primary, secondary and tertiary hemostasis, while primary and secondary hemostasis occur simultaneously. Primary hemostasis involves the activation of platelets and the formation of a platelet plug in order to cause a fast and primary arrest of bleeding, which is then strengthened during secondary hemostasis by a complex coagulation cascade. Cleavage of prothrombin results in the generation of prothrombin fragment 1 + 2 (F 1 + 2) and the serine protease thrombin, which is designated the key enzyme of the coagulation universe [5]. Next to mediating proteolytic cleavage of fibrinogen resulting in fibrin generation, the final step in the coagulation cascade, thrombin regulates endothelial cell function and activates procoagulant factors V, VIII, XI and XIII thereby accelerating coagulation [6,7]. Thrombin also mediates platelet activation via the protease-activated receptors (PAR) 1 and 4 as well as GPIb [8].

According to these reasons, thrombin plays a pivotal role in hemostasis and thrombosis. During cardiac surgery employing ECC, thrombin is indirectly inhibited by administration of heparin in concentrations ranging from 300-400 international units (IU) per kg body weight, whereas low-dose heparin is sufficient in ECMO or VAD [9–11]. Under

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physiological conditions thrombin is inhibited by the plasma serpin antithrombin-III (AT-III), whose inhibiting effect can be potentiated by therapeutic heparin administration, thereby intensifying the binding affinity of AT-III for thrombin. Due to the fast onset of action and easy neutralization using protamine, heparin represents the standard of care during ECC procedure.

In order to guarantee optimal heparinization of the patient and avoid perioperative complications, monitoring of blood coagulation during CPB is essential. Due to its practicability and fast performance, measurements of the activated clotting time (ACT) are routinely performed during cardiac surgeries in heparinized patients for determining the adequacy of anticoagulation [12]. Other monitoring techniques measuring blood clotting reactions include prothrombin time (PT), activated partial thromboplastin time (aPTT) and heparin management test (HMT) [9,13].

Due to its profound role, detection of free thrombin would be an interesting and reliable biomarker reflecting more precisely the actual hemostatic status during and after CPB procedures. However, its short half-life (1-2 min) [14], which is further decreased in the presence of heparin, may represent a major challenge in detection of free thrombin. Only recently, Müller et al. described for the first time a direct measurement of free active thrombin in patients using an aptamer-based biosensor system [15].

The aim of our study was to investigate the feasibility of measuring free active thrombin in heparinized plasma using an enzyme-capture-assay for the quantitative detection of active thrombin. We applied a well-established Chandler-loop model as well as fresh human whole blood anticoagulated with various heparin concentrations in the range of 0.75 to 3 IU/ml in order to mimic ECC as used during CPB, ECMO or VAD. Monitoring of free plasmatic thrombin was performed at various time points during 240 min of circulation and further compared to standardized enzyme-linked immunosorbent assays (ELISA) detecting the indirect thrombin markers prothrombin 1 + 2 (F 1 + 2) and thrombin-antithrombin-III (TAT) complexes. Furthermore, thrombin receptor expression on platelets, platelet activation and changes in cell count parameters were investigated before and during ECC.

Materials and Methods

Blood Sampling

Blood sampling procedures were approved by the ethics committee of the University of Tuebingen, Germany. Human whole blood was collected from healthy volunteers, who gave signed informed consent, and anticoagulated with heparin (0.75 or 1 IU/ml; Rathiopharm GmbH, Ulm, Germany). If higher heparin concentrations were needed, blood was additionally heparinized after sampling. The following exclusion criteria for the blood donators were strictly fulfilled: (i) smokers, (ii) intake of hemostasis-affecting agents like acetylsalicylic acid, oral contraceptives or non-steroidal antiphlogistics in the last 2 weeks before blood sampling.

Dynamic in Vitro Model

Fresh human whole blood, anticoagulated with either heparin (1 IU/ml) or Refludan (20 μg/ml) was filled in empty polypropylene tubes and treated with (i) PBS as control, (ii) collagen (20 μg/ml, Bio/Data Corporation, Horsham, USA) or (iii) kaolin (100 μg/ml; Sigma-Aldrich). Samples were then rotated for 30 min at 30 rpm and 37 °C.

In each experiment, untreated blood served for the measurement of baseline values without rotation. After blood taking or rotation, the blood was directly filled in tubes containing the corresponding terminating media. Plasma was prepared, shock frozen in liquid nitrogen and stored at -20 or -80 °C until further ELISA analyses.

Chandler-Loop Model

Polyvinyl chloride tubes (3/8" x 3/32"; noDOP, Raumedic, Muenchberg, Germany) of 50 cm length were filled with 20 ml of fresh heparinized blood.

Tubings were closed by a silicone tubing and rotated vertically at 30 rpm in a water bath at 37 °C for different time periods. For each donor, 20 ml of heparinized blood served for the measurement of baseline values before circulation in the Chandler-loop model. After blood taking or circulation, the blood was directly filled in tubes containing the corresponding terminating media or analyzed in flow cytometry. Plasma was prepared, shock frozen in liquid nitrogen and stored at -20 or -80 °C until further ELISA analyses.

Detection of Platelet Activation Using Flow Cytometry

Flow cytometric analyses of human platelets were performed directly after blood sampling (baseline) and after circulation. For evaluation of GPIIb/IIIa activation and GPIb expression on platelets, binding of the PAC-1 monoclonal antibody (mAb; BD Biosciences, Heidelberg, Germany) and the anti-GPIb mAb (Beckman Coulter, Marseille, France) were measured according to previously described methods [16,17].

For detection of PAR-1 expression, whole blood was diluted (1:50) in modified Tyrode's buffer and 50 μl of the suspension was incubated with 10 μl of an anti-PAR-1 mAb (Santa Cruz Biotechnology, Heidelberg, Germany) for 20 minutes in the dark.

Afterwards, samples were fixed using CellFix (BD Biosciences). For all samples, suitable isotype antibodies were used to adjust fluorescence amplification settings. A total of 10.000 events were acquired in each sample. Flow cytometry was performed within 6 hours using a FACScan cytometer (BD).

Measurement of Plasmatic Markers Indicating Thrombin Generation

The detection of plasmatic markers indicating thrombin generation was performed using different immunochemically tests. Conversion of prothrombin into active thrombin results in the generation of prothrombin fragment 1 + 2, which was measured using the Enzygnost® F 1 + 2 immunoassay according to manufacturer's instructions (Siemens Healthcare, Marburg, Germany).

Free plasmatic thrombin was detected using the OLIGOBIND® Thrombin activity assay (American diagnostica GmbH, Pfungstadt, Germany). Briefly, after each measurement blood was rapidly transferred into thrombin blood collection tubes containing citrate as well as the reversible thrombin inhibitor argatroban (American diagnostica GmbH) and directly centrifuged at 2500 g for 15 min. For thrombin detection, thrombin standards were prepared by the manufacturer by adding known amounts of purified α-thrombin to pooled citrated plasma containing argatroban. Standards and plasma samples were incubated in microwells coated with a bivalent DNA aptamer against thrombin. Following washing, a fluorogenic peptide substrate for thrombin was added and the change of fluorescence (360 nm extinction/460 nm emission) was measured over a time period of 30 min. False positive results obtained from detection of already complexed thrombin, like thrombin-alpha-2-macroglobulin complexes, are excluded by the manufacturer.

Inactivation of free thrombin was determined by measuring thrombin-antithrombin III complexes (TAT) with an enzyme-linked immunosorbent assay (Siemens Healthcare, Marburg, Germany).

Whole Blood Count Analysis

The number of erythrocytes, leukocytes and platelets as well as hematocrit and hemoglobin values were measured by a cell counter system (Axon Lab AG, Switzerland) in all baseline and circulated samples.

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