Thrombosis Research 158 (2017) 25–34

Contents lists available at ScienceDirect

Thrombosis Research

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

Full Length Article

Characteristics of thrombin generation in a fulminant porcine sepsis model

Judit Tóth [a], Ildikó Beke Debreceni [a], Ádám Deák [b], Katalin Pető [b], Mariann Berhés [c], Endre Hajdú [c], Judit Szabó [d], Norbert Németh [b], Béla Fülesdi [c], János Kappelmayer [a,*]

[a] Department of Laboratory Medicine, Faculty of Medicine, University of Debrecen, Debrecen, Hungary
[b] Department of Operative Techniques and Surgical Research, Institute of Surgery, Faculty of Medicine, University of Debrecen, Debrecen, Hungary
[c] Department of Anesthesiology and Intensive Therapy, Faculty of Medicine, University of Debrecen, Debrecen, Hungary
[d] Department of Medical Microbiology, Faculty of Medicine, University of Debrecen, Debrecen, Hungary

ARTICLE INFO

Article history:
Received 6 June 2017
Received in revised form 6 July 2017
Accepted 31 July 2017
Available online 01 August 2017

Keywords:
Platelet activation
Thrombin generation
Sepsis

ABSTRACT

Introduction: The activation of blood coagulation has been demonstrated in most cases of sepsis, however previous studies in humans could not detect hypercoagulability with global hemostasis assays. In a fulminant porcine sepsis model we analysed coagulation screening tests and thrombin generation to evaluate hemostatic alterations.

Materials and methods: Live *Escherichia coli* bacteria were inoculated to female pigs and prothrombin time, activated partial thromboplastin time, thrombin time and fibrinogen were measured by coagulometry. Platelet counts, platelet aggregates and platelet phosphatidyl serine (PS) expression were studied, furthermore in in vitro experiments the PS-inducing ability of septic and control plasmas was investigated by flow cytometry. Thrombin generation was carried out by the Ascent Fluoroscan reader and results were evaluated by the Thrombinoscope software.

Results: Clotting assays showed a large variability, but no systematic changes during the 4-hour observation period. Platelet count significantly decreased and the number of platelet aggregates increased already by 2 h compared to baseline values and to control animals. Although the increase in platelet PS expression was non-significant in the septic group, the septic plasma elicited PS expression on normal human red blood cells. Thrombin generation became significantly faster, but the quantity of formed thrombin demonstrated both hypo- and hypercoagulability depending on the setting of the assay.

Conclusions: Enhanced thrombin generation without activators and the PS-inducing capacity of septic plasma are signs of hemostatic activation during fulminant sepsis while the decreased amount of generated thrombin upon tissue factor and phospholipid induced activation demonstrates attenuated thrombin forming ability.

1. Introduction

Sepsis is a leading cause of death worldwide and an immense number of publications have contributed to our recent knowledge about the hemostatic changes that are observed in septicemia. Activation of blood coagulation has undoubtedly been documented in the majority of these studies, but most groups have failed to detect any hypercoagulability in septic patients. Contrary, by using global hemostasis assays including the thrombin generation test or thrombelastometry — like the ROTEM assay — several groups have demonstrated hypocoagulability in septic patients [1–3]. Thus, it comes of no surprise that in clinical studies therapeutic trials that aimed to neutralize thrombin like the administration of antithrombin, tissue factor pathway inhibitor and heparin have not proven to be effective, furthermore one potential candidate the activated protein C was also ineffective [4] and had to be removed from the market.

There has been a constant quest for finding evidence for the usefulness of the above mentioned global assays in clinical studies, however results were conflicting [1,5–9]. In a clinical setting the age, underlying disorders, clinical course of disease, administered therapy and high variability in the characteristics of infective agents may contribute to the variability of data observed in septic patients. Unlike in humans, all these factors can be standardized in animal models, thus results obtained in these studies may be more easily related to the causative agent. Three animal models became widely used: the peritonitis model elicited by cecal ligation and puncture [10], the induction of sepsis by endotoxin infusion [11] and the introduction of live bacteria to animals. Such

* Corresponding author at: Department of Laboratory Medicine, Faculty of Medicine, University of Debrecen, Nagyerdei krt. 98, 4032 Debrecen, Hungary.
E-mail address: kappelmayer@med.unideb.hu (J. Kappelmayer).

http://dx.doi.org/10.1016/j.thromres.2017.07.030
0049-3848/

experiments have been carried out also with Gram-positive bacteria [12], however the majority of these studies utilize Gram-negative bacteria — mostly *Escherichia coli* (*E. coli*) —, and there is a general agreement that endotoxin (lipopolysaccharide, LPS) injection serves rather as a model for endotoxin shock but is not an ideal model sepsis [13]. Another aspect of difference in platelet activation in LPS-infusion elicited endotoxemia induced experiments could be the type of LPS used. Not all LPS can induce platelet responses [14], thus, live bacteria may better mimic the in vivo pathological process in humans. In previous studies our group has optimized the conditions of a porcine septic model where live *E. coli* was introduced to pigs in a lethal sepsis model and was found to elicit early micro-rheological changes [15,16]. In recent studies we characterized hemostatic changes, as well as the kinetics of hypoxia induced alterations in relation to sepsis parameters and markers of organ damage during this fulminant sepsis model [17].

Since platelets are known to be involved in critical illness [18], in our study we set out to determine thrombin generation in platelet poor and in platelet rich porcine plasma samples, and simultaneously investigated classical hemostatic screening parameters. We have focused on the early events in this lethal sepsis model and intended to verify hemostatic activation by clotting assays, PS induction and various thrombin generation assays. The results demonstrated both hyper- and hypocoagulability and may add to our understanding about the pathophysiological changes that occur during severe sepsis.

2. Materials and methods

2.1. Porcine sepsis model

The experiments were carried in accordance with the European Community Guidelines and State Regulations with the approval of the University of Debrecen Committee of Animal Welfare (reg. Nr.: 21/2013. DEMAB). Seventeen juvenile female Hungahib pigs (bodyweight: 19.5 ± 1.6 kg) were subjected into septic (n = 10) and control groups (n = 7). Under general anaesthesia (15 mg/kg ketamine, i.m. + 1 mg/kg xylazine i.m.) tracheostomy was performed for assisted ventilation, and the left external jugular vein and the femoral artery have been cannulated for sampling and hemodynamic measurements. The animals did not receive anticoagulant or any medication.

In the *E. coli*-treated group, sepsis was induced by *E. coli* culture (2.5×10^5/mL; strain: ATCC 25922, Department of Medical Microbiology, University of Debrecen) suspended in physiological saline (API suspension medium, bioMérieux, Lyon, France) was intravenously administered in a continuously increasing manner according to the following protocol: 2 mL of bacterial culture suspended in physiological saline was injected in the first 30 min, then 4 mL in 30 min and afterwards 16 mL/h for 2 h. A total amount of 9.5×10^6 *E. coli* was infused within 3 h [15,16]. In the control group the similar volume of physiological saline solution was administered by the same protocol as in the septic group and no other intervention was made. The anaesthesia was maintained for 4 h and at the end of the experiment animals were euthanized.

2.2. Core temperature and hemodynamic parameters

The core temperature was measured via the central venous cannula by PiCCO Monitoring Kit (Pulsion Medical Systems SE, Germany) connected to Philips IntelliVue monitor (Koninklijke Philips N.V., The Netherlands). Heart rate and mean arterial pressure were also monitored invasively by the PiCCO device. The modified shock index (MSI) — which is a descriptive parameter for the circulation status and a clinically significant predictor of mortality in emergency patients — was calculated as the ratio of heart rate and mean arterial pressure values [19].

2.3. Blood drawing and sample preparation

Immediately before *E. coli* administration blood samples were collected from the cannulated vein directly into blood collection tubes (Becton Dickinson, San Jose, CA, USA) containing 0.105 M sodium-citrate as anticoagulant and the proportion of blood to the liquid anticoagulant volume was 9:1. The first 3 mL of blood was always discarded. Beside the baseline blood drawing, further blood samples were collected 2 and 4 h after starting the infusion containing *E. coli*. All samples were transported to the laboratory at 37 °C and the measurements were started within 20 min after blood drawing. Citrated whole blood was used for automated hematology analysis and blood smear examinations. Coagulation screening tests and thrombin generation were measured in platelet poor plasma (PPP). Citrated whole blood was centrifuged immediately at 1500*g* for 15 min at room temperature according to the manufacturer's instruction and this single-centrifuged PPP was used for coagulation screening tests and for the dilution of platelet rich plasma (PRP) to obtain a standard platelet count. PPP for thrombin generation assays was prepared by a second centrifugation at 10,000*g* for 10 min at room temperature according to Loeffen R et al. [20]. After centrifugation only the top two third of plasma was used. During flow cytometric measurements and also in a part of the thrombin generation studies, PRP was used which was obtained by centrifugation at 170*g* for 10 min at room temperature, then platelet count was determined by Sysmex XP 300 hematology analyser (Sysmex, Kobe, Japan) and was adjusted to 250 G/L by PPP.

2.4. Automated hematology analysis, light microscopy and coagulation screening tests

Blood cell count was performed according to our routine clinical laboratory procedure on ADVIA 120 hematology analyser (Siemens, Forcheim, Germany) using a special animal software suitable for porcine blood cell counting. Lobularity index (LI) value was also calculated by the same analyser. LI provides an indicator of the "left shift" of white blood cells (WBCs). During basophil/lobularity method of the analyser, red blood cells (RBCs) and platelets are lysed, then all WBCs except basophils — which remain intact — are stripped of their cytoplasm using a reagent and increased temperature. Based on shape and complexity of their nuclei, the analyser can categorize these stripped WBCs as mononuclear or polymorphonuclear cells. Baso X histogram displays the high-angle light scatter information (nuclear configuration) for this two WBC populations, therefore three different values are shown: mononuclear peak, mononuclear/polymorphonuclear valley and polymorphonuclear peak values. The LI is the polymorphonuclear peak value divided by 14, and decreased value indicates the appearance of immature WBCs in the circulation.

Platelet morphology was studied on peripherial blood smears using May-Grünwald and Giemsa staining. The microscopic examinations were carried out with Zeiss Axiostar Plus light microscope (Zeiss, Jena, Germany). The conglomeration of 3 or more platelets was defined as platelet aggregate and it was quantified as the number of platelet aggregates referred to 200 single platelets.

Coagulation screening tests (prothombin time, activated partial thromboplastin time (APTT), thrombin time and fibrinogen concentration) were determined by BCS coagulometer (Siemens, Forcheim, Germany) according to routine clinical laboratory procedures.

2.5. Flow cytometric measurements

Platelet PS expression was studied by an FC500 flow cytometer (Beckman Coulter, Brea, CA, USA) based on the modified method of Leytin V et al. [21] adapted to porcine platelets. PS expression was determined by annexin V binding to platelet surface using FITC-conjugated annexin V. Five microliter of PRP was stained with 5 μL of annexin V-FITC in 40 μL of annexin V binding buffer (both from BD Pharmigen,