Thrombosis Research 126 (2010) 337–344

Contents lists available at ScienceDirect

Thrombosis Research

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

Regular Article

Purified thromboplastin causes haemostatic abnormalities but not overt DIC in an experimental rabbit model

Line Olrik Berthelsen [a,*], Annemarie T. Kristensen [b], Mikael Tranholm [a]

[a] Novo Nordisk A/S, Måløv, Denmark
[b] Department of Small Animal Clinical Sciences, Faculty of Life Sciences, The University of Copenhagen, Denmark

ARTICLE INFO

Article history:
Received 4 March 2010
Received in revised form 31 May 2010
Accepted 29 June 2010
Available online 15 July 2010

Keywords:
Disseminated intravascular coagulation (DIC)
Animal model
Rabbit
Thromboplastin
Overt DIC

ABSTRACT

Validation of animal models of disseminated intravascular coagulation (DIC) to human DIC is crucial in order to translate findings in research models to treatment modalities for DIC in humans. ISTH classifications of overt and non-overt human DIC have proven to have a high diagnostic accuracy, and we have previously established a rabbit model of non-overt DIC based on the ISTH classification of non-overt DIC. In this rabbit model, we used purified rabbit brain thromboplastin to induce DIC and test applicability of ISTH classifications of overt human DIC.

Cardiovascular and haematological parameters from rabbits, either saline-injected or administered a 2.5 mg thromboplastin/kg bolus and a 15 minutes 1.25 mg thromboplastin/kg infusion, were determined at four time points over a 90 minute period. All groups of rabbits were scored at each time point according to the ISTH classifications of overt DIC.

Despite the fact that injection of purified thromboplastin resulted in decreased platelet count, increased prothrombin time, activated partial thromboplastin time, level of thrombin-antithrombin complexes and fibrin degradation products, and pulmonary micro-thrombosis, none of the rabbits were diagnosed as having overt DIC according to ISTH classification.

We conclude that purified thromboplastin causes haemostatic abnormalities in the rabbit but this experimental model was not diagnosed as overt DIC.

Disseminated intravascular coagulation (DIC) is a pathological generalised activation of the coagulation system occurring secondary to a wide spectrum of underlying diseases i.e. endotoxemia, tissue damage, or endothelial damage eventually leading to thrombus formation, bleeding and organ failure [1]. Although DIC is accompanied by pro-coagulant activation, fibrinolytic system activation, inhibitor consumption, cytokine release, cellular activation and end-organ damage, the degree to which these systems are activated varies according to the underlying disorder [2]. The International Society of Thrombosis and Haemostasis (ISTH) subcommittee on disseminated intravascular coagulation has categorised DIC into two stages with an associated scoring system: non-overt DIC, where the haemostatic system is stressed but compensated and overt DIC with a stressed and de-compensated haemostatic system [3]. Risk assessment of underlying disorders associated with DIC in combination with global tests of coagulation factors including fibrin degradation products (FDP), platelets, prothrombin time (PT) and fibrinogen scored as a group, constitute an internationally acceptable definition as well as diagnostic criteria to distinguish overt DIC from non-overt DIC in humans [3]. According to this overt DIC is defined by presence of an underlying risk condition, a platelet count $<100\times10^9$/L, prothrombin time (PT) prolonged >3 s, plasma fibrinogen concentration <1.0 g/l and elevated fibrin degradation products in plasma. According to ISTH and later validated by Toh et al. [4] characterization of non-overt DIC in man is based on the same parameters but with emphasis on the trend over time and measurements proceeding daily for an affirmative diagnosis in patients [3].

Animal models provide an important tool for the understanding of DIC, discovery of new treatment modalities for DIC and safety evaluation of new haemostatic components in development.

In previous rabbit models of DIC endotoxins have been the preferred inducer of DIC. The endotoxin-induced coagulation activation represents a typical clinical situation in patients with gram-negative sepsis. Endotoxin has been administered i.v. as a bolus [5], an infusion [6] or as i.v. bolus in combination with cortisone or a

Abbreviations: ISTH, International Society on Thrombosis and Haemostasis; DIC, disseminated intravascular coagulation; FDP, fibrin degradation product; PT, prothrombin time; AT, antithrombin; TAT, thrombin-antithrombin; PC, protein C; TEG, thromboelastography; FVIIa, activated factor VII; LAL, Limulus Amebocyte Lysate; BP, blood pressure; HR, heart rate; RVPmax, maximal right ventricular pressure; ECG, electrocardiogram; cTnI, cardiac troponin I; aPTT, activated partial thromboplastin time; ELISA, enzyme-linked immunoadsorbent assay; MA, maximal amplitude; R, clotting time; HE, hematoxylin-eosin; PTAH, phosphotungstic acid hematoxylin; WBC, white blood cell.

* Corresponding author. Hørdumsgade 15, 1.th, 2450 København SV, Denmark. Tel.: +45 40848869; fax: +45 44434537.
E-mail address: lineberthelsen@yahoo.dk (L.O. Berthelsen).

0049-3848/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.thromres.2010.06.022

preparative endotoxin injection [7]. Thromboplastin is also used for induction of DIC in animal models. Thromboplastin opposed to endotoxins exerts an immediate response with a short-lasting systemic thrombin generation [8]. The fast response to TF resembles the situation in certain diseases or disorders including malignancies [9], severe trauma [10] and obstetrical calamities [11], predisposing for DIC where tissue factor is believed to be the main initiator of DIC.

In order to use and translate findings in research models to treatment modalities for DIC in humans it is important to include the parameters involved in the ISTH scoring system in the measurements in the animal model system. However, since the publication of the human scoring system of DIC only a few animal models referring to this scoring system has been published [12]. The scoring system has successfully been applied to dogs suffering from non-overt and overt DIC for various reasons [13], but to our knowledge no experimental animal model of overt DIC as defined by the parameters of the scoring system has been reported.

In a previous study a thromboplastin induced model of non-overt DIC in the rabbit was established [14], however the thromboplastin was found to be contaminated with endotoxin. In an attempt to establish a model of overt DIC in the rabbit a lethal effect of high doses of thromboplastin forced us to discontinue the studies.

We hypothesized, that removal of the endotoxin from the thromboplastin would make it possible to establish a distinct tissue factor-induced rabbit model of overt disseminated intravascular coagulation (DIC) based on the diagnostic ISTH criteria including the 4 parameters mentioned. Laboratory tests such as thromboelastography (TEG) and analysis of TAT, Troponin I and right ventricular heart pressure were explored as new useful markers for sensitive and specific detection of overt DIC in its early phase.

Material and methods

Reagents

Individual vials of Thromboplastin rabbit brain (thromboplastin) 2 mg/ml were obtained (Thromboplastin rabbit brain, Fluka, Sigma-aldrich, Switzerland) and freshly dissolved in sterile physiological saline and pooled. The thromboplastin pool was characterized with regard to tissue factor concentration, found to be 2.46 nM, by a chromogenic assay using a known level of FVIIa – a modification of a previously decribed assay [15]. Also the endotoxin level was determined, in brief samples were diluted and lysated using lysate from horseshoe crab, Limulus Amebocyte Lysate (LAL) and added to a microplate. Endotoxin level was determined using a kinetic turbidimetric test performed with a temperature controlled plate reader Sunrise (TECAN, Switzerland). As a high endotoxin level was determined in the original thromboplastin vials (454 EU/ml), a purification step was added. Pooled vials were centrifugated at 4000 g for 2 min., the supernatant removed and the thromboplastin pool resuspended in sterile saline, which reduced the endotoxin contamination dramatically to a level <20 EU/ml, while preserving tissue factor and phospholipids. A tissue factor activity of 2.59 nM was determined in the purified thromboplastin, which equals a tissue factor level of 120 μg/ml in the thromboplastin solution. Presence of active phospholipids in the purified thromboplastin was verified in a prothrombinase assay, where prothrombinase activity was measured by a chromogenic substrate assay as previously described [16]. Addition of increasing concentrations of thromboplastin to a constant level of Factor Xa, Factor Va and prothrombin strongly enhanced the thrombin formation in this assay. Purified thromboplastin was furthermore shown to have a similar procoagulant effect on citrated whole rabbit blood as non-washed thromboplastin when tested in vitro by thromboelastography (TEG) (TEG is further described below).

Animals

Eleven female New Zealand white rabbits (Charles River, Germany) weighing 2.1–2.6 kg were included in the study.

The rabbits were housed in a barriered facility in colonies of 10 animals. A standard rodent pelleted diet (Altromin 2113) was fed ad libitum as well as fresh tap water. Animals were acclimated for a period of 1 week. The study was approved by the Danish Animal Experiments Inspectorate, the Ministry of Justice.

Animal preparation and instrumentation

Bodyweights were recorded and rabbits were pre-anaesthetized with Diazepam 5 mg/ml (Stesolid®, Alpharma, Oslo, Norway) 0.4 mg kg^{-1} i.v. in a marginal ear vein. Thereafter, pentobarbital sodium 5% in sterile water (Nomeco, Copenhagen, Denmark) was administered through the ear vein to effect and supplemented as needed. Furthermore an ear vein was catheterised for injection of test compound. Xylocain®, 10 mg/ml (Astra Zeneca, Denmark), was injected in the neck and a catheter (Polyethylene tubing PE200, BD, Broendby, Denmark) was placed in the left carotid artery for blood sampling and measurement of blood pressure (Physiological pressure transducer, ADinstruments Ltd., Oxfordshire, United Kingdom). The catheter was kept open by slow infusion of saline water 0.9%, 6.6 ml/hour. The right jugular vein was catheterised and the catheter advanced to the right heart ventricle for measurement of right ventricular heart pressure (Pressure catheter SPC-320, 2F polyurethane, connected to a mikro-tip BP foundation system, ADinstruments Ltd., Oxfordshire, United Kingdom). Core temperature was maintained at 38 °C using a homeothermic heating blanket with a rectal temperature probe (Homeothermic blanket system, Harvard Apparatus, Holliston, Massachusetts, USA).

Experimental design

In order to establish a model of experimental DIC a 2 min bolus injection of 2.5 mg thromboplastin/kg (mg thromboplastin/kg) at a volume of 2.5 ml/kg and an infusion of 1.25 mg thromboplastin/kg was administered i.v. at time point 0-2 minutes and 10-25 minutes respectively. Saline in the same volumes at the same time points was injected in control animals. Control animals were tested in between dosing groups.

Blood samples were collected before injection of test compound (-5 min) and at 5, 30, 60 and 90 minutes after bolus injection of thromboplastin (Fig. 1). Eight rabbits were enrolled in the dosing group. Three animals were enrolled as a control group.

Cardiovascular parameters

Systemic blood pressure was measured via the carotid catheter. Right ventricular pressure was measured by a pressure catheter advanced to the right heart ventricle via the jugular vein. An electrocardiogram was obtained and heart rate was determined from the electrocardiographic measurements. Hemodynamics and ECG variables were recorded continuously throughout the study.

Furthermore blood gas levels were measured at each time point using heparinised vials for immediate analysis (GEM Premiere 3000, ILS, Denmark).

Cardiac troponin I (cTnI) is a filament complex of cardiac muscle and a recognized biomarker of cardiomyocyte damage when measured in serum [17]. Serum levels of rabbit cTnI were determined by enzyme-linked immunosorbent assays validated for rabbit cTnI (Life Diagnostics, West Chester, PA).