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Serum des-R prothrombin activation peptide fragment 2: A novel prognostic marker for disseminated intravascular coagulation

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ABSTRACT

Introduction: Disseminated intravascular coagulation (DIC) is diagnosed based on the combination of predisposing underlying conditions and laboratory tests for plasma coagulation markers. Because the collection of blood plasma samples is a fastidious procedure, the serum sample method may be preferred for measurement of coagulation markers when feasible.
Materials and Methods: The novel serum marker des-R prothrombin activation peptide fragment 2 (des-R F2) was measured using a sandwich enzyme-linked immunosorbent assay in 181 patients suspected of having DIC. Thrombin generation potential was estimated with a calibrated automated thrombogram.
Results: Serum des-R F2 was generated with an *in vitro* clotting process within a serum separation tube after blood collection. Carboxypeptidase inhibitor inhibited the formation of des-R F2 during *in vitro* clotting. Low levels of prothrombin and thrombin generation potential resulted in low serum des-R F2 levels. Serum des-R F2 was significantly decreased in overt DIC. Levels of des-R F2 correlated with DIC severity and other coagulation markers. Of note, the decrease in serum des-R F2 levels was a significant marker for predicting mortality.
Conclusions: The serum marker, des-R F2, can be used for the investigation of DIC severity and prognosis. It should be considered a useful marker, especially when only serum samples are available.

Introduction

Disseminated intravascular coagulation (DIC) derives from the systemic activation of blood coagulation, which generates fibrin deposits, leading to microvascular thrombi in various organs and contributing to multiple organ dysfunction syndrome [1]. DIC is diagnosed based on the combination of predisposing underlying conditions and laboratory tests for routine coagulation markers such as prothrombin time (PT), activated partial thromboplastin time (aPTT), and fibrinogen [2,3]. In addition, several fibrin-related markers including D-dimer, serum fibrinogen/fibrin degradation product (FDP) and fibrin monomer have been reported [3–5]. We have also reported several plasma markers of DIC in earlier studies [6,7].

For accurate measurement of these coagulation markers, test samples should be collected in a tube containing 3.2% sodium citrate and plasma should be obtained after centrifugation within 4 h in order to prevent *in vitro* artifactual activation of the coagulation system. Given the relatively strict requirements for pre-analytic processes in plasma, serum sample may be preferred for the measurement of coagulation markers when feasible. Although serum FDP was developed for DIC marker, it needs to collect blood into special tube containing with fibrinolysis inhibitor [8]. To date, there have been no reports on DIC serum markers that do not need a special tube.

During coagulation activation, prothrombin is activated by the prothrombinase complex and prothrombin fragment 1.2 and thrombin are then produced [9]. The resulting fragment 1.2 is further divided into prothrombin activation peptide fragment 1 and prothrombin activation peptide fragment 2 (Fig. 1). The des-R prothrombin activation peptide fragment 2 (des-R F2) is generated after cleaving the final amino acid, arginine (R), from the carboxyl terminus of the prothrombin activation peptide fragment 2. Purification and identification of des-R F2 in human serum has been previously reported [10].

We hypothesized that des-R F2 is a potential serum marker for coagulopathy because it is produced during active coagulation processes. In this study, we investigated the clinical usefulness of serum des-R F2 as a prognostic marker for patients with DIC. Because plasma carboxypeptidase is known to cleave arginine from the carboxyl terminus

Abbreviations: aPTT, activated partial thromboplastin time; CI, confidence interval; des-R F2, des-R prothrombin activation peptide fragment 2; DIC, disseminated intravascular coagulation; ELISA, enzyme-linked immunosorbent assay; HR, hazard ratio; PT, prothrombin time; SE, standard error; TAFI, thrombin-activatable fibrinolysis inhibitor.

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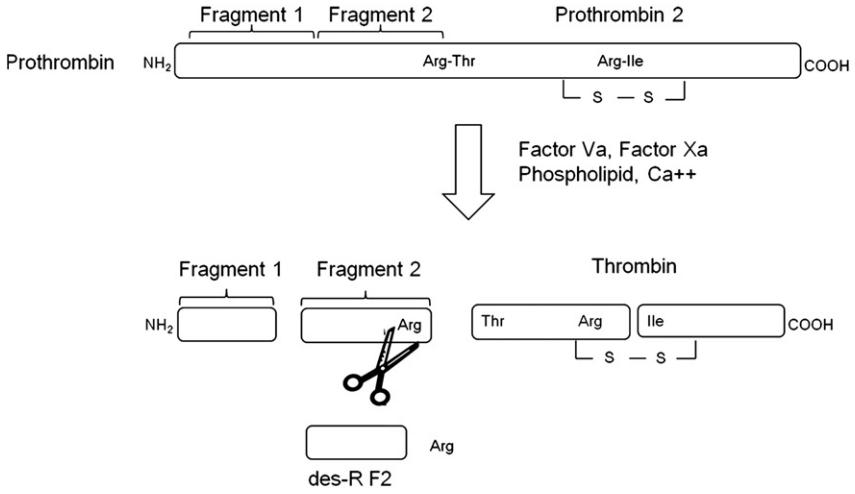

Fig. 1. Schematic of prothrombin activation. Human prothrombin is composed of fragment 1, fragment 2, and prothrombin 2. Prothrombinase complex (factor Xa, Va, phospholipid, and calcium ions) cleaves prothrombin into fragment 1, fragment 2, and thrombin. Subsequent arginine cleavage of fragment 2 at the carboxyl terminal produces des-R prothrombin activation peptide fragment 2 (des-R F2).

of proteins [11], we explored whether carboxypeptidase inhibitor can prevent the des-R F2 formation during *in vitro* clotting process.

Materials and Methods

Study Population

A total of 181 patients with suspected DIC who were scheduled for DIC screening were recruited. Exclusion criteria were thrombosis or bleeding disorders, warfarin or heparin use within 3 days of blood collection, or body weight <8 kg. In addition, 24 healthy adults were enrolled in order to determine the des-R F2 reference range. This study was approved by the Institutional Review Board of Seoul National University Hospital. Patients were diagnosed as having overt-DIC if they had a cumulative International Society on Thrombosis and Haemostasis subcommittee score of ≥5 [2]. We classified patients who did not meet overt DIC criteria (a cumulative score of <5) as "no overt-DIC."

Blood Sampling

Peripheral blood samples were collected in commercially available sodium citrate tubes (Becton Dickinson, San Jose, CA, USA) for coagulation tests and in serum separation tubes (Becton Dickinson). All blood samples were centrifuged for 15 min at 1550 × *g* after 2 h of collection. The supernatant plasma and serum were aliquoted and kept frozen at −70 °C.

For analysis of time-dependent *in vitro* generation of des-R F2, peripheral blood samples were aliquoted and kept frozen at serial times after blood drawing from healthy volunteers. In addition, peripheral bloods of healthy volunteers were collected in sodium citrate tube, heparin tubes and serum separation tubes (Becton Dickinson) simultaneously to investigate the anticoagulant effect on *in vitro* formation of des-R F2.

To explore the carboxypeptidase effect on *in vitro* des-R F2 formation, fresh peripheral bloods of healthy volunteers were incubated with or without carboxypeptidase inhibitor from potato tuber (final concentration 20 μg/ml; Sigma Aldrich, Saint Louis, MO, USA) and the resulting supernatants after 40 min incubation were aliquot and kept frozen as well.

Coagulation Assays

PT, aPTT, and fibrinogen levels were measured by the standard clotting assay on a STA-R analyzer (Diagnostica Stago, Asnières, France). Levels of D-dimer were measured by immunoturbidimetric assay performed on an ACL 3000 (Beckman Coulter, Fullerton, CA, USA). Antithrombin activity was determined by chromogenic assay (Stachrom ATIII, Diagnostica Stago). Protein C activity was also determined by chromogenic assay using an ACL 3000 (Beckman Coulter).

Thrombin generation was measured using a Fluoroscan Ascent fluorometer (Thermolabsystems, Helsinki, Finland) as described by Hemker et al. [12]. The thrombin generation assay (TGA) was performed in triplicates for all samples. The area under the thrombin generation curve that represented endogenous thrombin potential was used for test parameters.

Measurement of des-R F2

Serum des-R F2 level was measured using a home-made sandwich enzyme-linked immunosorbent assay (ELISA) assay. The coating antibody epitope was a des-R specific c-terminal portion (GLDEDSDRAIEG) of prothrombin fragment 2. The coating antibody was produced by the custom monoclonal antibody production service of GenScript (Piscataway, NJ, USA). A commercial prothrombin specific antibody (N77095M, BioDesign, Memphis, TN, USA) was used as a capture antibody after biotinylation. Six calibrators containing 0–80 ng/mL of des-R F2 and 2 controls containing low and high concentrations of des-R F2 were prepared by using recombinant des-R F2; this was custom-made by AbFrontier (Seoul, Korea). Briefly, after overnight coating with the coating antibody (2.5 μg/mL) in Immuno module (Nalge Nunc, International, Rochester, NY, USA) at 4 °C, we carried out a blocking process with phosphate buffered saline containing 5% skim milk. Serum samples were then diluted to 1:1000, and the calibrator and controls were incubated for 1 h at room temperature. After washing, the capture antibody (1 μg/mL) and horseradish peroxidase-labeled streptavidin(1:1000 dilution) (Bethyl Laboratories, Montgomery, TX, USA) were incubated sequentially. Horseradish peroxidase activity was measured using the TMB/sulfuric acid system (KPL, Gaithersburg, MD, USA). Mean absorbance values at 450 nm were used for construction of the calibration curve and determination of unknown des-R F2 concentrations. The intra-assay coefficient of variation was 2.5%, and the inter-assay coefficient of variation was 3.3%.

Statistical Analysis

All statistical analyses were carried out with SPSS 17.0 for Windows (SPSS Inc., Chicago, IL, USA). Continuous data comparisons were performed using the Mann–Whitney *U* tests and Kruskal–Wallis tests,