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Regular Article

Preserved clot formation detected by the Thrombodynamics analyzer in patients with cirrhosis

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ABSTRACT

Introduction: Patients with cirrhosis have substantial alterations in their hemostatic system, which are paradoxically associated with the risk of both bleeding and thrombotic complications. However, it still remains difficult to predict those risks, because results from conventional coagulation tests, such as the prothrombin time (PT) and activated partial thromboplastin time (APTT), do not reflect the complex hemostatic changes in these patients. More sophisticated global hemostasis tests, such as thrombin generation assays, are not standardized for routine use yet. Here we examined the spatial clot growth in plasma from patients with cirrhosis using the novel Thrombodynamics assay, which uses a fundamentally new approach to test plasma hemostatic capacity.
Materials and Methods: Thrombodynamics assays were performed in plasma from thirty-one patients with cirrhosis and twenty-five healthy controls. Results were compared to results with thrombin generation testing and PT/APTT test results.
Results: Rates of clot growth, clot size, and clot density from the Thrombodynamics assay were comparable between patients and controls. Thrombin generation in the presence of thrombomodulin was increased in the patients, despite prolonged PT and APTT test results. There was little correlation between parameters derived from the Thrombodynamics assay and the PT, APTT, or thrombin generation data.
Conclusions: The Thrombodynamics assay showed preserved clot formation in plasma from patients with cirrhosis, which is in line with the results of the thrombin generation assay in this study and previously reported by others.

Introduction

Conventional coagulations tests, such as the prothrombin time (PT) and activated partial thromboplastin time (APTT), are frequently prolonged in patients with cirrhosis suggesting a hypocoagulable state [1]. Traditionally, these findings have been considered as pointing towards a bleeding tendency in patients with a chronic liver disease. However, these coagulation tests are only sensitive for selected procoagulant factors and do not take the reduction in anticoagulant factors, which also occurs in these patients, into account. In fact, the hemostatic system in patients with a chronic liver disease is nowadays considered to be rebalanced [2]. However, this balance is more unstable compared to that in healthy individuals and there is thus a frequent clinical need for predicting the risk of bleeding or thrombosis in patients with a liver disease [2,3].

In contrast to the conventional coagulation tests, the thrombin generation test in the presence of thrombomodulin, the main activator of protein C, is sensitive to all anticoagulant systems in plasma and thus measures the true balance between the pro- and anticoagulant proteins. This test has shown normal to even increased thrombin generation in patients with a chronic liver disease [4–8]. However, the thrombin generation assay is not widely available yet. The test is currently too complicated for routine use in diagnostics laboratories, and the addition of thrombomodulin is not yet standardized.

Recently a new plasma-based global hemostasis assay, Thrombodynamics, has been developed that allows a continuous monitoring of clot growth in non-stirred plasma initiated by a thin layer of immobilized tissue factor (TF) [9,10]. This assay was designed to better mimic the *in vivo* conditions of clot formation by taking into account both the biochemical reactions of the coagulation cascade and the spatial aspects of clot formation. Indeed, while in other coagulation tests (e.g. PT and thrombin generation) clotting is activated by TF that is homogenously distributed over the plasma sample, in this test only a thin layer of plasma is exposed to TF and clot formation starts on this

Abbreviations: PT, prothrombin time; APTT, activated partial thromboplastin time; TF, tissue factor; HIV, human immunodeficiency virus; V_i, initial rate of clot growth; V_{st}, stationary rate of clot growth; PPP, platelet-poor plasma; SD, standard deviation; a.u, arbitrary units; ETP, endogenous thrombin potential; HCV, hepatitis C virus; NASH, non-alcoholic steatohepatitis; PSC, primary sclerosing cholangitis; NSAIDs, Nonsteroidal anti-inflammatory drugs; TM, thrombomodulin; R, spearman correlation coefficient

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surface and propagates into the bulk of plasma. This spatial clot growth assay previously showed defective clot formation (with lower rate of clot growth and thinner clots) in both patients with hemophilia A and B [11]. In addition, another study showed that spatial clot growth in plasma can be used to predict an increase in D-dimer levels in sepsis patients [9]. Furthermore, the test was useful for detecting procoagulant changes caused by an aptamer antagonist of tissue factor pathway inhibitor [10], recombinant factor VIIa [12], or platelet microparticles [13].

Here we aimed to examine the spatial clot growth in plasma from patients with a chronic liver disease using the novel Thrombodynamics assay and compared results with thrombin generation testing and PT/APTT test results.

Materials and Methods

Patients

Thirty-one adult patients with cirrhosis, who were seen on an outpatient basis or were admitted to the department of Hepatology of the University Medical Center Groningen, were included in the study. Patients were classified according to the Child-Pugh classification [14]. Eleven patients were classified as Child A, ten patients as Child B, and ten patients as Child C cirrhosis. Exclusion criteria were documented history of congenital coagulation disorders, presence of active infection (<2 weeks), presence of acute liver failure, use of anticoagulant drugs in the past 10 days, pregnancy, human immunodeficiency virus (HIV) positivity, and recent (<7 days) transfusion with blood products.

Twenty-five healthy volunteers working at our institute were included as controls. Exclusion criteria for the control group were a documented history of congenital coagulation disorders, documented history of hepatic disease, recent viral infection (<2 weeks), use of anticoagulant drugs in the past 10 days, pregnancy, and HIV positivity.

The study protocol was approved by the medical ethical committee of the University Medical Center Groningen, Groningen, The Netherlands and written informed consent was obtained from each subject before inclusion in the study.

Plasma samples

Blood samples from each patient and control were drawn by venapuncture and collected into vacuum tubes containing 3.8% trisodium citrate as an anticoagulant, at a blood to anticoagulant ratio of 9:1. Platelet poor plasma was prepared by double centrifugation at 2000 g and 10.000 g respectively for 10 min. Plasma was snap-frozen in liquid nitrogen and stored at -80 °C until use.

Thrombodynamics assay

The general concept of the Thrombodynamics test was previously described by others [9,10,15,16]. Briefly, in a thin layer of plasma coagulation is activated when it is brought in contact with tissue factor (TF) immobilized on a plastic surface. The clot formation starts on the activator and propagates into the bulk of plasma in which no TF is present. Light scattering by fibrin allows observation of spatial clot formation in real time by using time lapse imaging [17].

In this study, the Thrombodynamics assay was performed using an experimental device provided by HemaCore LLC (Moscow, Russia). Reagents (Thrombodynamics kit, Hemacore LLC, Mosow, Russia) and protocols from the manufacturer were used. According to these instructions, plasma was pre-treated with Corn Trypsin Inhibitor for 10 minutes at 37 degrees Celcius prior to initiation of the assay. The following parameters were analyzed: lag time, initial and stationary rates of cloth growth, clot density, and clot size at 30 minutes. The lag time is defined as the time between clotting initiation and actual appearance of the fibrin clot. The initial rate of clot growth (V_i) is the slope of the curve on a clot vs. time graph during the first 2-6 minutes of cloth growth. Stationary rate of clot growth (V_{st}) is measured as a slope of the curve on a clot size vs. time graph within the interval 15–25 minutes after clot growth begins.

Coagulation tests

Thrombin generation testing was performed using platelet-poor plasma (PPP) with the fluorimetric method described by Hemker, Calibrated Automated Thrombography® (CAT) [18]. Reagents and protocols were purchased from Thrombinoscope BV, Maastricht, The Netherlands. Coagulation was activated using commercially available reagents containing recombinant TF (final concentration 5 pM), phospholipids (final concentration 4 mM), in absence or presence of soluble thrombomodulin. To calibrate the thrombin generation curves, Thrombin Calibrator (Thrombinoscope BV) was added, and a fluorogenic substrate with CaCl2 (FluCa-kit, Thrombinoscope BV, Maastricht, The Netherlands) was used to allow a continuous registration of thrombin generation. Fluorescence was read in time by a fluorometer, Fluoroskan Ascent® (ThermoFisher Scientific, Helsinki, Finland).

The PT, APTT, and fibrinogen levels were assessed on an automated coagulation analyzer (ACL 500 TOP) with reagents (Recombiplastin 2G for PT, SynthaSil for APTT, and QFA thrombin (Hemosil) for fibrinogen) and protocols from the manufacturer (Instrumentation Laboratory, Breda, the Netherlands).

Statistical analysis

Data are expressed as means (with standard deviations (SDs)), medians (with interquartile ranges), or numbers (with percentages) as appropriate. Means of two groups were compared by Student's t-test or distributions in the two groups by Mann-Whitney U test as appropriate. Multiple groups were compared using one-way ANOVA (with the Bonferroni post test) or Kruskal-Wallis H test (with Dunn's post test) as appropriate. Spearman's correlation coefficient was used to assess correlation between continuous variables. P values of 0.05 or less were considered statistically significant. GraphPad Prism (San Diego, USA) and IBM SPSS Statistics 20 (New York, USA)) were used for analyses.

Results

Patient characteristics

The main characteristics of the study population are presented in Table 1. Thirty-one patients with cirrhosis (20 males and 11 females) were included, and they were categorized according to the severity of liver disease as expressed by the Child Pugh classes (11 Child A, 10 Child B and 10 Child C patients). Twenty-five healthy subjects (10 males and 15 females) with a mean age of 33.9 ± 11.1 (mean ± SD) were included as controls. The most common etiology of liver disease was alcoholic, especially in the Child class C patients. Three healthy subjects and none of the patients used oral contraceptives ($P = 0.08$). None of the healthy subjects and three patients used antiplatelet agents or nonsteroidal anti-inflammatory drugs (two used carbasalate calcium and one used naproxen) ($P = 0.25$).

Thrombodynamics assay

The results of the Thrombodynamics test in the plasma from patients and controls are presented in Table 2 and Fig. 1. The lag time was slightly, but significantly prolonged in the patients compared to the controls ($P = 0.025$). V_i was significantly increased in the Child class C patients (63.7 μm/min (59.4-68.6) (median with range)) compared to controls (57.0 μm/min (50.0-63.4); $P < 0.01$). However, the V_{st} was comparable between patients and controls. Clot size was also comparable between

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