Evidence of Normal Thrombin Generation in Cirrhosis Despite Abnormal Conventional Coagulation Tests

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The role played by coagulation defects in the occurrence of bleeding in cirrhosis is still unclear. This is partly due to the lack of tests that truly reflect the balance of procoagulant and anticoagulant factors *in vivo*. Conventional coagulation tests such as prothrombin time and activated partial thromboplastin time are inadequate to explore the physiological mechanism regulating thrombin, because they do not allow full activation of the main anticoagulant factor, protein C, whose levels are considerably reduced in cirrhosis. We used a thrombin generation test to investigate the coagulation function in patients with cirrhosis. Thrombin generation measured without thrombomodulin was impaired, which is consistent with the reduced levels of procoagulant factors typically found in cirrhosis. However, when the test was modified by adding thrombomodulin (*i.e.*, the protein C activator operating *in vivo*), patients generated as much thrombin as controls. Hence, the reduction of procoagulant factors in patients with cirrhosis is compensated by the reduction of anticoagulant factors, thus leaving the coagulation balance unaltered. These findings help clarify the pathophysiology of hemostasis in cirrhosis, suggesting that bleeding is mainly due to the presence of hemodynamic alterations and that conventional coagulation tests are unlikely to reflect the coagulation status of these patients. In conclusion, generation of thrombin is normal in cirrhosis. For a clinical validation of these findings, a prospective clinical trial is warranted where the results of thrombin generation in the presence of thrombomodulin are related to the occurrence of bleeding. (HEPATOLOGY 2005;41:553–558.)

See Editorial on page 434

Liver cirrhosis is characterized by a reduced capacity of the liver to synthesize coagulation factors.[1-3] In addition, some patients with cirrhosis show hyperfibrinolysis[4-6] or, less frequently, chronic intravascular coagulation,[7] which may combine to further reduce plasma coagulation factors. These complex defects can be documented through the measurement of coagulation factors which are, with the only exception of factor VIII,[8] below normal limits, or through the prolongation of such global tests as the prothrombin time (PT) and the activated partial thromboplastin time (APTT). While portal hypertension is recognized as the main cause of bleeding in patients with cirrhosis,[9] the role played by coagulation defects in the occurrence of bleeding is still unclear. This may reflect a partial association between the severity of bleeding and the degree of coagulation abnormalities,[10] as well as the fact that conventional coagulation tests fail to reflect blood coagulation as it occurs *in vivo*.

Coagulation and fibrin formation may be conveniently seen as a two-sided coin (Fig. 1). The first side is the procoagulant drive triggered by tissue factor when this cellular receptor forms a complex with plasmatic factor VII,[11] which in turn ignites a series of reactions ultimately leading to thrombin generation and fibrin clot formation.[12] The other side is the anticoagulant drive originating from thrombin itself, which, once complexed with its endothelial receptor thrombomodulin, activates plasmatic protein C.[13] Activated protein C is a potent anticoagulant that, in combination with its cofactor protein S, downregulates thrombin generation through the inactivation of the activated forms of factor VIII and factor V.[13] The anticoagulant drive is also potentiated by the presence of plasma antithrombin,[14] which, in complex with

Abbreviations: PT, prothrombin time; APTT, activated partial thromboplastin time; ETP, endogenous thrombin potential; FU, fluorogenic unit.

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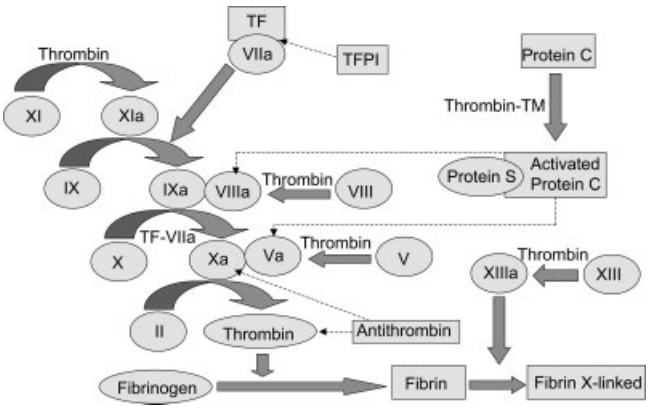

Fig. 1. Simplified scheme of the coagulation cascade. **Roman numbers** represent coagulation factors. **Solid and broken arrows** represent procoagulant and anticoagulant mechanisms, respectively. TF, tissue factor; TFPI, tissue factor pathway inhibitor; TM, thrombomodulin.

endothelial heparin-like substances, inhibits thrombin directly through the formation of an equimolar complex and indirectly through the inactivation of such activated coagulation factors as XI, IX, and X.[15] Thrombin formation is also downregulated by the tissue factor pathway inhibitor that specifically inhibits the complex tissue factor: factor VII[16] (Fig. 1).

The balance between the procoagulant and anticoagulant drives is essential to ensure unwanted thrombin generation in physiological conditions. This balance is usually investigated by means of laboratory tests such as the PT and APTT that are based on the rate of conversion of fibrinogen to fibrin. A limitation of conventional laboratory tests is that plasma starts to clot soon after as little as 5% of the whole thrombin is generated, thus leaving the remaining 95% undetected.[17] In addition, because these tests are performed without added thrombomodulin, they are inadequate to explore the physiological mechanisms regulating thrombin formation because they do not allow full activation of protein C. This activation is 20,000 times slower than that attained in the presence of thrombomodulin.[18] The recent development of newer tests based on the continuous registration of thrombin generation under *in vitro* conditions that mimic more closely what occurs *in vivo*[19] prompted us to reinvestigate the balance between procoagulants and anticoagulants in patients with cirrhosis.

Patients and Methods

Patients

Forty-four adult patients with cirrhosis (31 men, 13 women) with a median age of 52 years (range, 24-79) were enrolled in the study, which was approved by the local ethics committee. The diagnosis of cirrhosis was based on clinical, laboratory, and ultrasound evidence. Criteria of exclusion from the study were the use of medications known to affect blood coagulation, recent bleeding (within the last 6 months), bacterial infection, hepatocellular carcinoma, extrahepatic malignancy, and known hemostatic disorders other than liver disease. Severity of cirrhosis was estimated according to Child-Turcotte-Pugh classification.[20] Forty-four healthy subjects comparable for age, sex, and geographical origin with the patients volunteered to participate in the study as controls. After informed consent, blood was drawn without stasis by clean venepuncture and collected in vacuum tubes containing 105 mmol/L trisodium citrate as an anticoagulant (Vacutainer; Becton Dickinson, Meylan, France) at a blood-anticoagulant ratio of 9:1. Blood was centrifuged within 30 minutes at controlled room temperature for 15 minutes at 2,000*g*. Plasma was then harvested and filtered through 0.22-μm cellulose acetate filters (Millipore, Bedford, MA) to eliminate residual platelets. The platelet-free plasma was subsequently aliquoted in plastic-capped tubes, quickly frozen in liquid nitrogen, and stored at -70°C until it was tested for thrombin generation (see Methods), which was performed no later than 6 months after blood collection. Aliquots of nonfiltered plasma were frozen as outlined above and used for conventional coagulation testing (see Methods). Aliquots of blood were also collected from patients and controls to prepare serum samples for the measurement of the biochemical parameters employed to assess liver function.

Methods

Thrombin Generation. Thrombin generation was measured as endogenous thrombin potential (ETP) according to Hemker et al.[21] and as described in detail by Chantarangkul et al.[22] The test is based on the activation of coagulation in the test plasma after addition of human relipidated recombinant tissue factor (Recombiplastin; Instrumentation Laboratory, Orangeburg, NY) suitable to replace conventional thromboplastin in the PT test,[23] which acts as a coagulation trigger in the presence of synthetic phospholipids as a platelet substitute (DOPS, DOPE, and DOPC in the proportion of 20:20:60 [M/M]; Avanti Polar Lipids Inc., Alabaster, AL). The concentrations of tissue factor and phospholipids in the test system were 1 pmol/L and 0.5 μmol/L, respectively. In some experiments, soluble thrombomodulin (ICN Biomedicals, Aurora, OH) was added in the reaction mixture at a final concentration of 4 nmol/L. Continuous registration of the generated thrombin was achieved with a fluorogenic synthetic substrate (Z-Gly-Gly-Arg-AMC HCl, Bachem, Bubendorf, Switzerland) added to the test system at a final concentration of 2.5 mmol/L. The entire procedure was performed with an automated fluorometer

Table 1. Demographic Characteristics of Study Population Separated According to Child-Turcotte-Pugh Classes

Characteristics	All (n = 44)	Child-Turcotte-Pugh Class A (n = 14)	Child-Turcotte-Pugh Class B (n = 16)	Child-Turcotte-Pugh Class C (n = 14)	*P* Value
Age (yr)	55 (24–79)	58 (42–79)	60 (40–77)	51 (24–71)	.08
Sex (male)	34	11	12	11	.96
Etiology					.43
Alcoholic (n)	12	1	6	5	
HCV (n)	18	9	6	3	
HBV (n)	7	2	3	2	
Other (n)	7	2	1	4	
Serum bilirubin (mg/dL)	2.0 (0.6–25)	1.2 (0.6–2)	1.9 (0.7–3.8)	4.2 (2.3–25)	<.001
Prothrombin time (ratio)*	1.27 (0.94–1.81)	1.15 (0.97–1.27)	1.30 (0.94–1.70)	1.49 (1.24–1.81)	<.001
Serum albumin (g/L)	34 (18–47)	40 (31–47)	34 (22–40)	27 (18–34)	<.001
Serum creatinine (mg/dL)	0.8 (0.5–2.4)	0.9 (0.5–1.2)	0.75 (0.6–1.2)	0.7 (0.5–2.4)	.86
Child-Turcotte-Pugh score	9 (5–13)	5.5 (5–6)	8.5 (7–9)	11 (10–13)	<.001
MELD score	9.4 (1.8–26.7)	6.6 (1.8–9.9)	8.5 (12.3–12.7)	12.5 (8.8–26.8)	<.001
Hemoglobin (g/dL)	11.4 (8.1–15.6)	13.9 (10.4–15.6)	11.3 (8.1–15)	10.8 (8.4–14)	.002
Leucocytes (10^9/L)	4.1 (1.25–9.30)	3.9 (1.3–6.8)	4.6 (1.25–8.7)	3.8 (2.4–9.3)	.83
Platelets (10^9/L)	69 (24–215)	76 (24–199)	89 (39–215)	52 (25–136)	.11
Bleeding time (min)	6.5 (2–20)	6 (3–18)	7.25 (2–17)	7 (4–20)	.88

NOTE. Data are presented as median (range) or absolute numbers.

Abbreviations: HCV, hepatitis C virus; HBV, hepatitis B virus; MELD, model for end-stage liver disease.

*Patient-to-normal coagulation time.

(Fluoroskan Ascent; ThermoLabsystem, Helsinki, Finland) capable of simultaneously handling several samples in a completely automated fashion. Readings from the fluorometer were automatically recorded by a dedicated software (Thrombinoscope; Synapse b.v., Maastricht, The Netherlands), which displays thrombin generation curves (time vs. generated thrombin) and calculates the area under the curve, defined as ETP and expressed as fluorogenic units (FU) per minute. This is the whole amount of thrombin that can be generated by test plasma under the specified conditions and represents the balance between the action of procoagulants and anticoagulants in plasma. To minimize methodological variability, equal numbers of test plasmas from patients and controls were tested on each working session.

Other Coagulation Parameters. The PT was measured with human relipidated recombinant thromboplastin (Recombiplastin, Instrumentation Laboratory) in combination with a fully automated photo-optical coagulometer (ACL, Instrumentation Laboratory). Results were expressed as ratios of test to reference plasma. By definition, the greater the ratio, the longer the coagulation time for the test plasma. The reference plasma was prepared by pooling equal amounts of plasma from 30 healthy donors (15 men and 15 women), which was subdivided in small aliquots in plastic-capped tubes, quick-frozen in liquid nitrogen, and stored at −70°C. The APTT was measured with the automated APTT reagent (bioMerieux, Durham, NC), and results were expressed as ratios of test to reference plasma. Factor II (prothrombin) activity was measured using S2238 (Instrumentation Laboratory) as the chromogenic substrate and Echis Carinatus (Sigma, St. Louis, MO) as the activator.[24] The test was performed on an automated coagulometer (Electra 1600C, Instrumentation Laboratory). Factor VIII activity was measured with standard APTT-based coagulation assay with factor VIII–deficient plasma[25] on an automated coagulometer (Electra 1600C, Instrumentation Laboratory). Protein C and antithrombin were measured as functional activities by the commercial methods Proclot Protein C (Instrumentation Laboratory) and Electrachrome Antithrombin (Instrumentation Laboratory). Results for all factor measurements were expressed as a percentage of the pooled normal plasma (see previously in this paragraph) arbitrarily set at 100% activity. All the other tests, which included the measurement of the biochemical parameters to assess liver function, were performed with standard laboratory techniques.

Statistical Analysis. Continuous variables were expressed as medians and ranges. The nonparametric Mann-Whitney *U* test was used to test for differences between median values where appropriate. Spearman coefficient of correlation was used to assess for correlation between different variables. *P* values of .05 or less were considered statistically significant. All analyses were performed with SPSS version 11.5 software (Chicago, IL).

Results

The main characteristics of the patient population are reported in Table 1. The distribution of severity of liver disease according to Child-Turcotte-Pugh classification

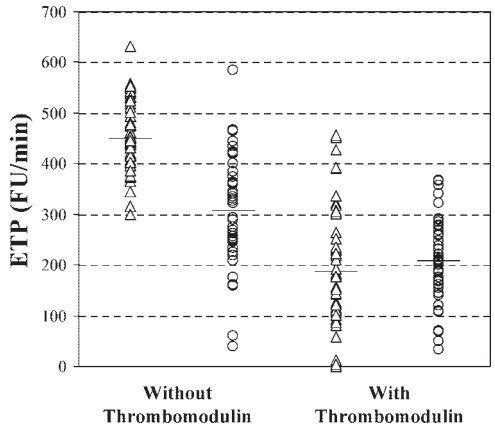

Fig. 2. ETP values for controls **(triangles)** and patients **(circles)** measured without and with thrombomodulin. **Horizontal bars** represent median values. Abbreviations: ETP, endogenous thrombin potential; FU, fluorogenic unit.

identified three groups of similar size. Figure 2 shows the results of thrombin generation expressed as ETP (FU/min) for patients and controls. When the test was performed without thrombomodulin, the median ETP was 308 FU/min (range, 40-585) for patients and 451 FU/min (range, 300-632) for controls ($P < .001$), indicating that less thrombin was generated in patients with cirrhosis. The ETP values in patients were negatively correlated with model for end-stage liver disease score ($r = -0.66$; $P < .001$) and Child-Turcotte-Pugh score ($r = -0.55$; $P < .001$). When the test was performed with thrombomodulin, the median ETP was 209 FU/min (range, 34-368) for patients and 188 FU/min (range, 0-455) for controls. These values were not significantly different ($P = .50$). ETP values with and without thrombomodulin were positively correlated both for patients ($r = 0.53$; $P < .001$) and controls ($r = 0.42$; $P < .01$).

The results of the other coagulation measurements are shown in Table 2. Median PT and APTT ratios were significantly higher in patients with cirrhosis than in controls ($P < .001$), whereas factor II, antithrombin, and protein C levels were significantly lower ($P < .001$). The ratio between factor II and protein C levels, taken as a rough index of the balance between the pro- and anticoagulant drives responsible for thrombin generation, was significantly higher in patients with cirrhosis than in controls ($P < .001$). All the above measurements were correlated to Child-Turcotte-Pugh score (PT, $r = 0.68$, $P < .001$; APTT, $r = 0.53$, $P < .001$; protein C, $r = -0.67$, $P < .001$) and model for end-stage liver disease score (not shown). Median factor VIII levels tended to be higher in patients with cirrhosis than in controls, but the difference did not reach statistical significance ($P = .14$).

Discussion

In spite of the fact that most coagulation factors are low in patients with cirrhosis, the impairment of the hemostatic function in these patients is poorly understood from a mechanistic perspective. Patients with cirrhosis rarely bleed in comparison with patients with the same degree of congenital or acquired coagulation deficiencies; when they do bleed, the sites and types of bleeding are different (gastrointestinal bleeding vs. soft tissue hematomas and hemarthroses). To understand the reason for such a difference, a thorough investigation of the coagulation system in these patients is required. However, available tests do not entirely mimic the process of thrombin generation as it occurs *in vivo*, and particularly the balance between the procoagulant and the anticoagulant drives; tests such as PT and APTT are responsive only to procoagulant factors. Activated protein C, the main anticoagulant mechanism that downregulates thrombin generation *in vivo*, is hardly reflected by these tests because the time interval during which they explore coagulation is not sufficient for thrombin to significantly activate plasma protein C and hence the anticoagulant pathway. Because plasmatic levels of protein C are considerably reduced in patients with cirrhosis, we surmise that to be meaningful, coagulation tests should be designed to reflect the balance between procoagulant and anticoagulant factors.

In this study, we used a test designed to investigate thrombin generation in order to assess the function of blood coagulation in patients with cirrhosis. The results show that thrombin generation measured without thrombomodulin is reduced (Fig. 2), which is consistent with the reduced levels of zymogen factor II (prothrombin) that are typically found in these patients. On the other hand, when the test was modified by adding thrombomodulin to allow full activation of protein C, patients with liver cirrhosis generated nearly as much thrombin as control subjects (Fig. 2), suggesting that in patients with

Table 2. Hemostatic Parameters in Patients With Liver Cirrhosis (n = 44) and in Controls (n = 44)

Parameters	Patients	Controls	*P* Value
PT (ratio)*	1.26 (1.02-2.53)	0.99 (0.89-1.18)	< .001
APTT (ratio)*	1.31 (0.95-4.00)	0.99 (0.80-1.19)	< .001
Protein C (%)†	39 (9-77)	105 (79-142)	< .001
Antithrombin (%)†	52 (16-94)	101 (76-112)	< .001
Factor II (%)†	49 (16-81)	105 (84-130)	< .001
Factor II/protein C (ratio)‡	1.28 (0.78-2.43)	1.00 (0.63-1.33)	< .001
Factor VIII (%)†	132 (43-446)	124 (65-223)	.14

NOTE. Data are presented as median (range).
*Patient-to-normal coagulation time.
†Percentage activity relative to the reference normal plasma set at 100%.
‡Factor II-to-protein C activity.

cirrhosis the reduction of factor II (procoagulant drive) is balanced by the reduction of protein C (anticoagulant drive) (Fig. 1), thus leaving the coagulation balance unaltered. Indeed, the ratios between factor II and protein C levels in our patients showed that protein C is reduced to a greater extent than factor II (Table 2). This greater degree of reduction of the anticoagulant compared with the procoagulant factors might explain the rather mild or absent tendency of patients with cirrhosis to bleed from sites other than the gastrointestinal tract. A similar mechanism has been demonstrated in neonates that, despite having markedly decreased levels of vitamin K–dependent coagulation factors, have a normal coagulation balance.[26] Other factors may be responsible for the normal thrombin generation shown by the modified test in patients with cirrhosis. Factor VIII, a known procoagulant factor, is increased in these patients compared with controls (Table 2). The role played by high factor VIII in enhancing thrombin generation is plausible if one considers that high levels of this factor have been associated previously with increased levels of such biochemical markers of thrombin generation as the prothrombin fragment 1 + 2.[27] Antithrombin (Table 2) and the tissue factor pathway inhibitor,[28] the other two naturally occurring anticoagulant mechanisms operating in plasma, are lower than normal in patients with cirrhosis; furthermore, they may help to restore a coagulant balance and to avoid bleeding other than that caused by portal hypertension and related hemodynamic alterations.

This study has some limitations. Even though we applied a test system that reflects what occurs *in vivo* more closely than any global conventional coagulation test, the assay conditions are only partially physiological. Platelets have been replaced by synthetic phospholipids. Platelet-rich plasma would be a more physiological medium, but its preparation would be impractical. On the other hand, using platelet-poor plasma supplemented with exogenous phospholipids as a platelet substitute is common practice even for conventional coagulation tests. Thrombomodulin *in vivo* is located on the surface of endothelial cells, whereas in our system it is in the fluid phase. Furthermore, because thrombomodulin is located on endothelial cells, any inference about the physiological concentration to be used in the test is difficult. The final concentration used in this study (4 nmol/L) was drawn from previous estimates based on the assumption of the density distribution of this receptor on endothelial cells lining human arteries.[18] On the other hand, all the aforementioned limitations apply to patients as well as controls investigated in this study, making it unlikely that the pattern of similar thrombin generation in patients and controls is influenced by the test conditions to a different extent in the two groups.

In summary, the findings that stem from this study help to improve our knowledge on the pathophysiology of hemostasis in patients with cirrhosis, suggesting that bleeding events are mainly due to hemodynamic alterations secondary to portal hypertension and that the derangement of coagulation may not play a major role. Perhaps these findings also help to explain the relatively small clinical efficacy of such a powerful procoagulant agent as recombinant-activated factor VII in the control of variceal bleeding.[29,30] However, prospective clinical trials relating the results of the thrombin generation test, and the conventional PT and APTT to the occurrence of bleeding events are warranted before the new mechanistic information provided by this study can be applied to the clinical field. Another potential clinical implication of these findings is that the laboratory investigation of the coagulation function, presently performed with the PT and APTT, may be inadequate to assess the true risk of bleeding when patients with cirrhosis undergo invasive procedures such as liver biopsy and transplant surgery. Perhaps the measurement of thrombin generation in the presence of thrombomodulin might be more suitable to evaluate the hemorrhagic risk. Although plausible, this hypothesis needs to be substantiated clinically by a prospective study.

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