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Normal to increased thrombin generation in patients undergoing liver transplantation despite prolonged conventional coagulation tests

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Summary

Background & Aims: Patients with liver disease often show substantial changes in their hemostatic system, which may aggravate further during liver transplantation. Recently, thrombin generation in patients with stable disease was shown to be indistinguishable from controls provided thrombomodulin, the natural activator of the anticoagulant protein C system, was added to the plasma. These results indicated that the hemostatic balance is preserved in patients with liver disease, despite conventional coagulation tests suggest otherwise.

Methods: Here we examined thrombin generation profiles in serial plasma samples taken from ten consecutive patients undergoing liver transplantation.

Results: At all time points, the endogenous thrombin potential (ETP) was slightly lower compared to healthy volunteers, despite substantially prolonged PT and APTT values. However, when thrombin generation was tested in the presence of thrombomodulin, the ETP was equal to or even higher than that in healthy subjects. In fact, thrombin generation was hardly affected by thrombomodulin, while thrombin generation in healthy subjects decreased profoundly upon the addition of thrombomodulin. In patients undergoing liver transplantation, efficient thrombin generation in the presence of thrombomodulin may be explained by decreased levels of protein C, S, and antithrombin, and by elevated levels of FVIII.

Conclusions: Thrombin generation in patients undergoing liver transplantation is equal or even superior to thrombin generation in healthy volunteers when tested in the presence of exogenous thrombomodulin. These results support the recently advocated restrictive use of plasma during liver transplantation and warrants further study of the prophylactic use of anticoagulants to reduce thromboembolic complications after transplantation.

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Keywords: Coagulation; Liver transplantation; Thrombin; Thrombomodulin; Prothrombin time.
Introduction

Cirrhosis of the liver may result in substantial changes in the hemostatic system including thrombocytopenia and reduced platelet function, and decreased levels of pro-coagulant and anti-fibrinolytic proteins [1]. Although these alterations result in a reduced prohemostatic capacity, they are all, at least to a certain extent, balanced by compensatory factors. The reduced platelet count and function are balanced by highly increased levels of the platelet adhesive protein von Willebrand factor [2]. Reduced levels of pro-coagulant proteins are balanced by reduced levels of natural anticoagulant proteins [3], and the reduced levels of anti-fibrinolytic proteins are balanced by reduced levels of profibrinolytic proteins [4].

Traditionally, the hemostatic changes in patients with liver disease are thought to result in a hemostatic-dependent bleeding tendency [5]. However, recent clinical and laboratory data have challenged this theory which has led to the concept of ‘rebalanced hemostasis’ in patients with liver disease [6–8]. The hemostatic capacity of patients with liver disease appears to be preserved, but the balance is more easily disturbed as compared to healthy individuals. Indeed, patients with liver disease can present with hemostasis-related bleeding episodes, but may also be at risk for developing thromboembolic complications [9,10]. These thrombotic complications even occur in patients with liver disease while they are being treated with anticoagulant drugs [9].

The concept of rebalanced hemostasis has been elegantly exemplified by examinations of the thrombin generating capacity of plasma from patients with liver disease [3]. Thrombin generation is considered an essential step in the generation of a fibrin clot, and is traditionally assessed in routine clinical practice by the prothrombin time (PT) and activated partial thromboplastin time (APTT). In patients with liver disease, both PT and APTT are frequently prolonged, and the prolongation is proportional to the severity of the disease. Thrombin generation, as tested by calibrated automated thrombography (CAT) was shown to be decreased in patients with liver disease, in line with prolonged PT values. However, when a soluble form of
the endothelial transmembrane protein thrombomodulin, which is involved in activation of the natural anticoagulant protein C, was added to the test mixture, thrombin generation in patients with stable liver disease was indistinguishable from that in healthy volunteers [3]. In other words, thrombin generation tests performed under conditions in which the natural anticoagulant systems are fully activated revealed a completely preserved thrombin generation in patients with (end-stage) liver disease.

CAT results in thrombin generation profiles which are characterized by an initial increase in thrombin generated in time, followed by a decline in thrombin generated as a result of the actions of the natural anticoagulant systems. A widely used parameter to estimate total thrombin generating capacity is the area under the thrombin generation curve, or endogenous thrombin potential (ETP) [11]. This parameter was previously used to estimate thrombin generation in patients with liver disease [3]. However, additional parameters derived from the thrombin generation curve, specifically the peak height, the time required to reach the peak, and the initial rate of thrombin generation may give relevant additional information.

In patients with cirrhosis, additional changes in the hemostatic system occur during liver transplantation [12]. These alterations are traditionally believed to contribute to the bleeding tendency during this major surgical procedure [13]. However, transfusion requirements during liver transplantation have dropped tremendously during the last decade, and a substantial proportion of patients can now undergo this lengthy procedure without the requirement for any blood transfusion [14,15]. Although many factors including better surgical and anesthesiological techniques have contributed to the decline in transfusion requirements, a liver transplantation in a truly coagulopathic patient (e.g., a patient with hemophilia) would probably never be possible without factor replacement or transfusion. The large proportion of patients that can nowadays undergo a liver transplantation without transfusion requirement, suggest that the hemostatic potential is in balance during liver transplantation even though the conventional coagulation tests suggest otherwise. In fact, although bleeding was a
major concern during liver transplantation in the past, nowadays the concern has shifted towards the risk of thrombotic complications in the post-operative period [8]. Liver-related thrombosis, such as thrombosis of the hepatic artery or portal vein may occur following liver transplantation, but also systemic thromboembolic complications such as intracardiac thrombosis and pulmonary embolism have been reported [16]. Some investigators have suggested that a dysregulated hemostatic system may play a role in the occurrence of these potentially devastating thromboembolic complications [17,18].

Here we aimed to examine the thrombin generating capacity in patients undergoing liver transplantation. Serial plasma samples were taken during and after liver transplantation to perform a detailed analysis of several parameters derived from the thrombin generation curves in the absence and presence of thrombomodulin, and to examine changes in the anticoagulant pathways.

**Materials and methods**

**Patient characteristics**

Ten adult patients undergoing a liver transplantation between July 2007 and March 2008 who gave written informed consent were included in this study. The study protocol was approved by the Medical Ethical Committee of the University Medical Center Groningen, The Netherlands. Median age was 55 (range 25–60), and four were female. Patients received a transplant for primary sclerosing cholangitis (n = 2, one retransplant for recurrent disease 6 years after the initial transplant), cryptogenic cirrhosis (n = 2, one retransplant for recurrent disease 3 years after the initial transplant), hepatitis C, autoimmune hepatitis, acute liver failure, alcoholic cirrhosis and hepatocellular carcinoma (HCC), non-alcoholic steatohepatitis and HCC, and erythropoietic protoporphyria complicated by biliary cirrhosis. The mean model for end-stage liver disease score was 18 (range 11–27). Nine patients received red blood cell transfusion, and six of them required more than two units. Fresh frozen plasma was administered in four patients, and platelet concentrates in two.
Blood samples

Blood samples were taken at the following time points during and after surgery: 30 min after induction of anesthesia, 30 min after the start of the anhepatic phase, 30 min after reperfusion, at the end of surgery, and at days 1, 5, and 10 after surgery. Blood samples were obtained from a dedicated non-heparinized arterial line and were drawn into 3.2% sodium citrate (9:1, v/v). To obtain platelet-poor plasma, samples were centrifuged twice at 1000g for 10 min, after which the samples were stored at _80 °C until use. Individual plasma samples from 40 healthy volunteers from our laboratory were used to establish reference values for the ETP parameters. Individual plasma samples from 60 healthy volunteers from our laboratory were used to establish reference values for protein C and S, antithrombin, and FVIII. Pooled normal plasma was obtained by combining plasma from 200 healthy volunteers from our laboratory.

Assays

Coagulation assays prothrombin time (PT) and activated partial thromboplastin time (APTT) were performed on an automated coagulation analyzer (Behring Coagulation System, BCS) with reagents and protocols from the manufacturer (Siemens Healthcare Diagnostics, Marburg, Germany). Total protein S antigen was assayed by ELISA using antibodies from DAKO (Glostrup, Denmark). Free protein S was measured by precipitating the C4b-binding protein-bound fraction with polyethylene glycol 8000 and measuring the concentration of free protein S in the supernatant. Protein C was determined using the Coamatic protein C activity kit from Chromogenix (Mölndal, Sweden). Antithrombin activity was determined with Berichrom Antithrombin (Siemens Healthcare Diagnostics, Marburg, Germany). Factor VIII antigen levels were determined using the FVIII Asserachrom Assay (Diagnostica Stago, Asnieres Sur Seine, France). Levels are given as percentages relative to pooled normal plasma.

The Calibrated Automated Thrombogram assays the generation of thrombin in clotting plasma using a microtiter plate reading fluorometer (Fluoroskan Ascent, ThermoLab systems, Helsinki, Finland) and Thrombinscope software (Thrombinscope BV, Maastricht, The Netherlands). The assay was carried out as
described by Hemker et al. [11] and the Thrombinoscope manual. Coagulation was triggered by recalcification in the presence of 1 pM recombinant human tissue factor (Innovin, Siemens Healthcare Diagnostics, Marburg, Germany), 0.8 IM phospholipids, and 417 µM fluorogenic substrate Z-Gly-Gly-Arg-AMC (Bachem, Bubendorf, Switzerland). Fluorescence was monitored using the Fluoroscan Ascent fluorometer (ThermoLab systems, Helsinki, Finland), and the ETP, peak, time-to-peak, lag time and velocity index were calculated using the Thrombinoscope® software (Thrombinoscope, Maastricht, The Netherlands). The peak and velocity index were normalized with pooled normal plasma from 200 healthy volunteers as described previously [19].

The effect of thrombomodulin on thrombin generation was tested by the addition of rabbit lung thrombomodulin (2 nM, American Diagnostica, Greenwich, CT) to the plasma. The ETP was then determined as described above. A normalized ratio (TM-SR) was determined by dividing the ETP in the presence of thrombomodulin of an individual by the ETP in the presence of thrombomodulin of pooled normal plasma. A TM-SR > 1 reflects a decreased anticoagulant response to thrombomodulin in comparison to pooled normal plasma.

**Statistical analysis**

Statistical analysis was performed using the GraphPad InStat software package (GraphPad, San Diego, CA). Differences in ETP parameters were examined by standard one-way analysis of variance (ANOVA) using the Dunnett post-test. In these analyses, values were compared either with the group of healthy volunteers, or with values measured at the start of surgery. P values less than .05 were considered statistically significant.

**Results**

*Tissue factor-induced thrombin generation in platelet-poor plasma taken during and after liver transplantation*

We estimated the thrombin generating capacity of plasma by assessment of various parameters derived from the thrombin reduced total thrombin generating capacity,
the time to peak was slightly, but not significantly shorter in patients at the start of surgery compared to controls (Fig. 1A). Furthermore, the time to the peak of the thrombin generation curve progressively shortened during surgery and returned towards normal levels at postoperative day 1. The velocity of thrombin generation was similar in patients at the start of surgery compared to controls (Fig. 1E). However, at post-operative day 5 and 10, the velocity index was substantially elevated. Similarly, the maximal thrombin concentration generated (peak thrombin) was similar in patients at the start of surgery compared to controls, and was substantially elevated at post-operative day 10 (Fig. 1F). However, after reperfusion and at the end of surgery, the thrombin peak was significantly lower compared to healthy volunteers.

At multiple time points during and after surgery, the ETP was decreased compared to healthy volunteers despite a comparable or even increased velocity index and peak thrombin in patients compared to healthy volunteers, which is explained by a more generation curve generated using platelet-poor plasma in which thrombin formation was initiated by tissue factor and phospholipids. At the start of surgery, the ETP was slightly but significantly decreased compared to values observed in healthy volunteers (Fig. 1A). The ETP dropped substantially, although not significantly compared to the preoperative values after reperfusion, and increased again at post-operative day 1. Despite the reduced total thrombin generating capacity, the time to peak was slightly, but not significantly shorter in patients at the start of surgery compared to controls (Fig. 1D). Furthermore, the time to the peak of the thrombin generation curve progressively shortened during surgery and returned towards normal levels at postoperative day 1. The velocity of thrombin generation was similar in patients at the start of surgery compared to controls (Fig. 1E). However, at post-operative day 5 and 10, the velocity index was substantially elevated. Similarly, the maximal thrombin concentration generated (peak thrombin) was similar in patients at the start of surgery compared to controls, and was substantially elevated at post-operative day 10 (Fig. 1F). However, after reperfusion and at the end of surgery, the thrombin peak was significantly lower compared to healthy volunteers.
Fig. 1. Parameters derived from thrombin generation curves at various time points during and after liver transplantation, compared with values found in 40 healthy volunteers. Thrombin generation was induced by addition of tissue factor (1 pM) and phospholipids (0.8 nM) and was assessed in absence (panels A, D, E, and F) or presence (panels B, C, G, H, and I) of 2 nM thrombomodulin. (A) Endogenous thrombin potential, (B) endogenous thrombin potential in the presence of thrombomodulin, (C) thrombomodulin sensitivity ratio, (D,G) time-to-peak, (E,H) normalized velocity (pooled normal plasma set at 100%), (F,I) normalized peak (pooled normal plasma set at 100%). *p < 0.05, **p < 0.01 compared to controls. +p < 0.05, +++p < 0.01 compared to start of surgery.

At multiple time points during and after surgery, the ETP was decreased compared to healthy volunteers despite a comparable or even increased velocity index and peak thrombin in patients compared to healthy volunteers, which is explained by a more rapid decay in thrombin generation after the peak thrombin has been reached in patients. In other words, the width of the thrombin generation curve is smaller in patients as compared to healthy volunteers. The concomitant decrease in pro- and
anticoagulant factors in patients results in a relative increase in anticoagulant potency, a phenomenon that has been recognized previously in models of hemodilution (reviewed in [20]).

**Defective regulation of thrombin generation by thrombomodulin in plasma taken during and after liver transplantation**

Total thrombin generation in plasma from healthy volunteers was substantially decreased upon addition of soluble thrombomodulin (ETP without TM: 1752 nM min, range [987–2665], mean ETP with TM: 761 [125–1766], p < 0.0001). However, thrombin generation in patients before liver transplantation was only minimally affected by thrombomodulin (Fig. 1B). Moreover, during and after surgery the ETP was similar in all samples in the presence or absence of thrombomodulin. In fact, thrombin generation in the presence of thrombomodulin was significantly elevated compared to healthy volunteers at the start of surgery, in the anhepatic phase, and at post-operative days 5 and 10, whereas after reperfusion and at the end of surgery thrombin generation in the presence of thrombomodulin was not significantly different from that of healthy volunteers.

When these data were recalculated to a thrombomodulin sensitivity ratio, it became evident that at all time points during and after liver transplantation, thrombomodulin was ineffective at regulating thrombin generation (Fig. 1C). To substantiate that
thrombomodulin was indeed ineffective at regulating thrombin generation in patients undergoing liver transplantation, we also evaluated the time to peak, velocity index, and peak heights, which were essentially not altered by addition of thrombomodulin, with the exception of the normalized peak values, which were slightly higher in the presence of thrombomodulin (Fig. 1G, H, and I).

**Apparent intact thrombin generating capacity is not reflected by PT and APTT values**

At the start of surgery, both PT and APTT were substantially prolonged compared to normal reference values (Fig. 2). After reperfusion, both PT and APTT were extensively

![Graphs showing plasma levels of protein C, total protein S, antithrombin (AT), and factor VIII at various time points during and after liver transplantation. The large horizontal lines represent the upper and lower limit of reference values of each parameter, which was determined in healthy laboratory volunteers. Small horizontal lines represent medians.](image)

**Fig. 3.** Plasma levels of protein C, total protein S, antithrombin (AT), and factor VIII at various time points during and after liver transplantation. The large horizontal lines represent the upper and lower limit of reference values of each parameter, which was determined in healthy laboratory volunteers. Small horizontal lines represent medians.
prolonged, even to an extent that no clot formation was observed during the course of the assay in some samples. PT and APTT reduced towards normal levels at post-operative day 1. When excluding the samples in which no clot formation in PT or APTT was observed, a reasonable correlation between ETP without thrombomodulin and PT \((r = 0.55, p < 0.0001)\) or APTT \((r = 0.41, p = 0.015)\) was observed.

**Partial deficiencies of the natural anticoagulants and persistently elevated levels of FVIII may explain the apparent intact thrombin generating capacity in the presence of thrombomodulin**

We subsequently examined plasma levels of the natural anticoagulants protein C, protein S, and antithrombin during and after liver transplantation (Fig. 3). Levels of all three proteins were substantially decreased compared to reference values at the start of surgery (all approximately 50% of normal), and progressively decreased during surgery. Nadir levels were around 25% of normal and levels of all proteins started to recover towards normal levels at post-operative day 1. Also, free protein S levels were decreased (data not shown), as these showed a strong correlation with protein S levels \((r = 0.91, p < 0.0001)\). Factor VIII levels were substantially elevated at the start of surgery, dropped slightly towards normal values during surgery, but were substantially elevated post-operatively until day 10. FVIII levels correlated well with VWF antigen levels \((r = 0.54, p < 0.0001)\).

**Discussion**

This study shows remarkable dysregulation of thrombin generation in plasma samples taken during and after liver transplantation. Most notably, samples taken during and after liver transplantation were without exception profoundly resistant to thrombomodulin, the physiological activator of the natural anticoagulant protein C. Whereas in healthy individuals thrombin generation substantially decreased when thrombomodulin was added to plasma samples, essentially no effect of thrombomodulin was observed in plasma samples taken from patients undergoing
liver transplantation. Consequently, thrombin generation in the presence of thrombomodulin was higher than that of healthy volunteers at the start and during the anhepatic phase of surgery, and at post-operative days 5 and 10. Furthermore, peak thrombin generation and the velocity of thrombin generation were significantly higher in the post-operative period. These results provide evidence that the coagulation potential in patients with endstage liver disease undergoing liver transplantation is normal or even elevated, compared to healthy controls. These findings reinforce the recent notion that the hemostatic system in patients with cirrhosis is sufficiently competent to allow a large surgical procedure such as liver transplantation without the requirement for (major) blood transfusion [14,15]. In fact, these patients may be at an increased risk for post-operative thrombosis as a result of this hypercoagulable status.

The intact thrombin generating capacity before and during liver transplantation is not reflected by the routine assays that are used to test for hemostatic competence in clinical practice. In fact, the PT and APTT are substantially prolonged from the start of surgery up until day 5, although thrombin generation in the presence of thrombomodulin is higher or equal to thrombin generation observed in healthy volunteers. This confirms that the PT does not give clinically relevant information in patients with complex alterations in the hemostatic system, as the PT and APTT are only sensitive for levels of pro-coagulant proteins [21]. It is therefore also questionable whether the PT and APTT should be used to guide plasma transfusion during liver transplantation. As we have argued previously, the therapeutic efficacy of plasma transfusions in patients with liver disease have not yet been convincingly shown [22]. In fact, plasma transfusions may even contribute to bleeding as a result of volume overload resulting in exacerbation of portal hypertension, and by augmentation of anticoagulant proteins.

The thrombomodulin resistance during surgery is in part explained by the decreased levels of protein C and its cofactor protein S. Combined with the low levels of antithrombin this results in normal or even supranormal thrombin generation during or after liver transplantation, despite decreased levels of procoagulant proteins. The
reason for the persistent thrombomodulin resistance in the post-operative period is 
less clear, as the levels of protein C and S were almost normalized at post-operative 
day 5, which would allow for a normalization of thrombomodulin-mediated inhibition 
of thrombin generation. However, levels of factor VIII remain substantially elevated, 
and since high levels of factor VIII have been shown to induce APC resistance in an 
ETP-based test, this is likely an explanation for the persistent thrombomodulin 
resistance after liver transplantation. Indeed, when we spiked factor VIII deficient 
plasma with purified factor VIII, we observed a dose-dependent increase in TM-SR, 
which reached a plateau of a TM-SR of around 1.5 at 400% of factor VIII (data not 
shown).

Our results are in line with previous observations made by Tripodi and co-workers, 
who demonstrated that thrombin generation in patients with stable liver disease is 
similar to that in healthy volunteers provided thrombomodulin is added to the 
reaction mixture [3]. In contrast to our results, Tripodi et al. showed that addition of 
thrombomodulin reduced thrombin generation in patients with liver disease, albeit to 
a lesser extent than in control subjects. These investigators, therefore, also found that 
plasma from patients with cirrhosis is thrombomodulin resistant, but not to the extent 
as was observed in the current study. This small discrepancy in results may be explained 
by slight differences in experimental conditions. Most notably, the source of 
thrombomodulin differed between the studies (human vs. rabbit).

It has to be noted that our thrombin generation tests were executed in the absence of 
platelets. Tripodi and co-workers also showed that in those patients with a low platelet 
count, thrombin generation in the presence of thrombomodulin was slightly decreased 
compared to healthy volunteers, who had substantially higher platelet levels [23]. 
Platelet count in our patients was also substantially decreased compared to reference 
values (median 55 G/l, range 24–223), and thus thrombin generation in vivo may be 
somewhat lower than suggested by the results in this study. Nevertheless, it is clear 
from our data that the coagulation capacity in patients undergoing liver
transplantation is not as poor as traditionally believed based on conventional coagulation tests such as PT and APTT.

The post-operative increased velocity and peak levels in thrombin generation curves likely reflect the hypercoagulable status in the early post-operative period which has been described previously [24]. These results, combined with the recently described post-operative dysbalance in the von Willebrand factor/ADAMTS13 axis [25], strongly suggest that patients after liver transplantation have a hypercoagulable status in both the primary and the secondary hemostatic system.

In conclusion, thrombin generation in samples taken during and after liver transplantation is equal or superior to thrombin generation in healthy volunteers in the presence of exogenous thrombomodulin, which may be attributable in part to a profound thrombomodulin resistance. These results support the recently advocated restrictive use of plasma during liver transplantation. Furthermore, the results of our study support exploration of more extensive use of anticoagulants in the post-operative period to reduce the incidence of potentially devastating thromboses of the hepatic artery or portal vein.

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References
