Comparison of coagulation and thrombin generation in the portal and jugular plasma of patients with cirrhosis

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Summary

Portal vein thromboses are frequent in cirrhotic patients and may be favoured by hypercoagulability in the splanchnic venous system. The coagulation balance and thrombin generation (TG) were evaluated in platelet-free plasma obtained from portal and systemic blood samples in 28 cirrhotic patients while undergoing transjugular intrahepatic portosystemic shunt. TG assay (TGA) was performed with all samples from cirrhotic patients and with plasma samples from 14 healthy controls, with varying concentrations of tissue factor and phospholipids, with or without thrombomodulin. Screening tests and specific assays were also performed and activated partial thromboplastin time was shorter in portal plasma samples with higher FVIII and lower protein C levels, well correlated with Child-Pugh scores, and higher D-dimers and F1+2 levels. However, all TGA parameters were similar in portal and jugular samples, possibly due in part to similar concentrations of factor II and antithrombin in these two sites of plasma sampling. TGA showed lower thrombin peaks and endogenous thrombin potential values in cirrhotic plasma compared to those of healthy controls. Importantly, a resistance to thrombomodulin that well correlated with factor VIII and PC levels, was evidenced in all samples from patients with cirrhosis, and was more significant in those from severely affected cases. This study therefore supports the existence of a relative hypercoagulability in the portal vein of cirrhotic patients that is likely due to protein C/S deficiency and to high FVIII levels.

Keywords
Cirrhosis, thrombin generation assay, liver disease, coagulation

Introduction

Cirrhosis, a common end stage of chronic liver disease, results from the necrosis of liver cells and fibrosis with nodule formation. The distorted liver architecture interferes with liver function and blood flow, and thereby leads to impaired liver cell function and portal hypertension. Coagulation defects are therefore very frequent in severe cirrhosis and contribute to the occurrence of bleeding episodes in affected patients (1). Conventional coagulation laboratory assays such as prothrombin time (PT) and activated partial thromboplastin time (APTT) are widely used to evaluate the risk of bleeding before invasive procedures or surgical interventions. However, these methods are not fully effective to evaluate the haemostatic procoagulant/anticoagulant balance in liver cirrhosis, which is also characterised by a combination of defects in coagulation inhibitors (i.e. antithrombin, protein C, protein S) in the most severe cases that may contribute to the development of thrombosis in the portal venous system (2).

New methods that are able to provide a more global assessment of haemostasis have recently been evaluated in cirrhotic patients. One of these procedures, the thrombin generation assay (TGA), allows continuous measurement of thrombin formation during coagulation triggered by tissue factor (TF) (3–5). In addition, TGA appears promising to investigate the haemostatic balance overall, and its potential usefulness has recently been emphasised in both prothrombotic (6–9) and haemorrhagic clinical conditions (10, 11, 12). Interestingly, when evaluated by TGA, the generation of thrombin was recently found to be similar in adult patients with cirrhosis and in normal controls when the test was performed after addition of thrombomodulin (13). Moreover, it was also demonstrated that TGA was able to evaluate the role of platelets in thrombin generation in patients with cirrhosis (14).

For some patients, the treatment of portal hypertension requires the use of a transjugular intrahepatic portosystemic shunt (TIPS) (15, 16), and the procedure allowing the insertion of a TIPS provides the opportunity for blood sampling in the portal

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vein system. This possibility therefore prompted us to perform a comparative evaluation of the coagulation in peripheral and portal blood in a series of patients with cirrhosis in order to improve our understanding of the pathogenesis of portal thrombosis in this particular clinical situation.

Patients and methods

Patients and controls

Twenty-eight cirrhotic patients (mean age 58 years, range 37–76 years, 24 males and 4 females) due to undergo implantation of a transjugular intrahepatic portosystemic shunt (TIPS) were included. The principal aetiology of cirrhosis was alcoholism (n=24), 3 patients had non-alcoholic steatohepatitis and one had alcoholic cirrhosis with hepatitis C. The indications for implantation of a TIPS were refractory ascites (n=14), prevention of recurrent varical bleeding (n=12), refractory hydrothorax (n=4) and/or portal thrombosis (n=3).

The severity of the disease was evaluated by the Child-Pugh score (17). Nine patients were classified as Child-Pugh A (range of scores = 5–7), 11 patients as Child-Pugh B (range of scores = 7–9) and eight were scored Child-Pugh C (range of scores = 10–12).

TGA was also studied in the plasma of peripheral venous blood collected from 14 healthy men (mean age 25 years, range 20–45), non-carriers of FV Leiden and FII 20210A mutations and with normal haemostasis parameters.

All subjects were informed of the aims of the study and gave written consent.

Blood sampling and preparation of platelet-poor plasma

The insertion of TIPS was performed as previously described (16). Whole blood was collected in the jugular vein after being punctured and after discarding the first millilitres of blood. A catheter was then advanced through the right atrium and usually the right hepatic vein into a portal branch under ultrasound guidance in order to avoid multiple attempts at puncture. Another blood sample was then collected at the splenomesenteric junction before the TIPS implantation or any other gesture. Portal and jugular plasma sample was then collected after discarding the first millilitres of blood. A catheter was then advanced through the right atrium and usually the right hepatic vein into a portal branch under ultrasound guidance.

Screening tests and specific haemostasis assays

Prothrombin time (PT), factor II (FII) and factor V (FV) levels were measured using Neoplastin CI® and FII- and FV-deficient plasma (Stago, Asnières France). Activated partial thromboplastin time and factor VIII (FVIII) clotting activity were assayed using CK-prest® (Stago) and FVIII-deficient plasma obtained from Siemens (Marburg, Germany). Plasma fibrinogen (Fg) levels were measured by the Von Claus method using FibriQuick® reagent from Trinity Biotech (Champigny sur Marne, France). Protein C (PC), protein S (PS) and antithrombin (AT) plasma levels were measured by automated functional assays (Staclot PC®, Staclot PS® and Stachrom AT®, respectively, all from Stago). All tests were carried out in an automated coagulometer (STAR®, Stago).

Plasma levels of tissue factor (TF), D-dimers and prothrombin fragment 1+2 (F1+2) were measured by ELISA methods i.e. Immu-bind® Tissue factor from American Diagnostica, (Neuville/Oise-France), D-dimer Exclusion® Vidas from BioMerieux, (Craponne-France) and Enzygnost® F1+2 from Siemens. In addition, free TFPI levels were also measured in 18 patients in both jugular and portal plasma by ELISA (Asserachrom® Free TFPI, Stago).

FV Leiden and Prothrombin G20210A polymorphisms

Genomic DNA was extracted using a commercial Kit (flexigene DNA Kit®) from Quiagen (Courtabeuf, France). Factor V Leiden (G1691A) and factor II G20210A mutations were detected using a multiplex polymerase chain reaction-directed mutagenesis protocol (18).

Thrombin generation assay

Thrombin generation assay (TGA) was performed with the CAT reagent according to Hemker et al. (3, 19) using the following reagents: recombinant relipidated tissue factor (TF), i.e. Innovin®, was obtained from Siemens. Phospholipids (PL) were purchased from Avanti Polar lipids (Albaster, AL, USA) and PL vesicles consisting of 20% phosphatidylserine, 60% phosphatidylcholine and 20% phosphatidylethanolamine were prepared by a rapid extrusion procedure (20). The mixing buffer contained 20 mM Hepes (Sigma, St. Louis, MO, USA), 140 mM NaCl and 5 mg/ml bovine serum albumin (Sigma), pH 7.35. Soluble rabbit lung thrombomodulin (TM) (American Diagnostica Inc.) was obtained from Hyphen Biomed (Neuville sur Oise, France) and chondroitinase from Sigma. The fluorogenic substrate (Z-gly-Gly-Arg- AMC) and calcium in hepes buffer (FluCa-Kit®) and the calibrator (thrombin calibrator®) were commercial preparations purchased from Stago.

All TGAs were performed with an automated fluorometer (Fluoroscan Ascent® fluorometer Thermolabsystems) equipped with a dispenser (Biodis, Signes, France). Briefly, 80 μl of each plasma

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tested were dispensed into round bottomed microtiter plates (Greiner, Poitiers, France). Twenty μl of a mixture containing TF and PL were added to the plasma sample in the absence or presence of soluble TM. Two procedures with different final concentrations of reagents were used. Procedure A was performed using final concentrations of 5 pm FT and 4 μM PL in the absence or presence of 15 nM TM (21). Procedure B was carried out with lower final concentrations of reagents (i.e. 1 pm TF and 1 μM PL) with or without 4 nM TM (6). Finally, 20 μl of a starting reagent containing the fluorogenic substrate and CaCl2 were added. Measurement lasted 60 min and the reading interval was 20 seconds (s). Two wells were used to test each plasma sample, one to measure thrombin generation and the other for the calibration. All experiments were carried out in triplicate. Portal and jugular plasma samples from each patient were always studied in the same experiment. In addition, the same control plasma was tested in all experiments in order to correct day-to-day variations as defined below. This control plasma was collected from a young healthy man previously identified as being a non-carrier of FV Leiden or FII 20210A mutation and prepared and stored as described above for patients’ PPR. All coagulation parameters of this plasma sample were normal (PT 81%, APTT ratio 1.12, FII 80%, FV 60%, Fg 2.50g/l, FVIII 60%, PC 110%, PS 80%, AT 100%, D-dimers 96 ng/ml).

The molar amount of thrombin generated in the plasma was calculated by dedicated software (Thrombinoscope™ version 3.0.0.29, Thrombinoscope BV, Maastricht, The Netherlands), which automatically displays thrombin generation curves (nM thrombin vs. time in min). The duration of the lag-phase that followed the addition of the trigger (lag time in min), the thrombin peak (nM) and the endogenous thrombin potential (ETP, nM*min) corresponding to the area under the curve, were calculated for each assay.

ETP was corrected for day to day variations according to Tchai-kovski et al. (22), first by dividing the ETP obtained with the plasma sample by the ETP of the control plasma determined in the same experiment, and then by multiplying the result obtained by the reference value defined as the average of all ETP values of control plasma determined within the study. The influence of TM was assessed by calculating the normalised TM sensitivity ratio (nTMSR) = corrected ETP with TM / corrected ETP without TM) / (mean control ETP with TM/ mean control ETP without TM).

Statistical analysis

Continuous variables were expressed as median and range values and the non-parametric Wilcoxon signed rank test for paired values was used to investigate differences between portal and jugular blood. Correlations between numeric variables were evaluated using the non-parametric Spearman rank test. Differences in numeric variables between patients and controls or according to Child-Pugh scores were assessed by the non-parametric Mann-Whitney U test (Statview® software).

Results

Screening tests and specific haemostasis assays

The median and extreme values of PT, APTT, FII, FV, FVIII and fibrinogen plasma levels and plasma activity of AT, PC and PS measured in portal and jugular blood samples are presented in Table 1.

There were no differences between prothrombin time, FII, FV and fibrinogen levels in the jugular and portal blood of the 28 patients studied, and each of these parameters varied according to the intensity of the hepatic failure evaluated by the Child-Pugh score. In contrast, APTT ratios were significantly lower in the portal venous compartment (median = 1.23 vs. 1.30 in jugular blood, p = 0.004) and FVIII levels were also significantly higher (94% vs. 15% in jugular blood, p < 0.0001). In addition, the difference in FVIII level was significant whatever the Child-Pugh score, despite the limited number of patients in each category (Table 1). On the other hand, similar plasma levels of TF were measured in jugular and portal blood and also slightly increased with the severity of liver failure. Free TFPI levels measured in 18 patients also appeared identical in jugular (median = 13; range 9–31) and portal plasma (median = 11.5; range 7–28 ng/ml).

Coagulation inhibitors, FV Leiden, FII 20210A mutations and markers of hypercoagulability

None of the patients was a carrier of the FV Leiden G1691A mutation and one subject was heterozygous for the FII G20210A mutation.

Levels of AT were similar in jugular and portal samples and well correlated with the Child-Pugh score (p < 0.0004, and 0.002, respectively). In contrast, PC and PS levels were significantly lower in portal plasmas than in those measured in jugular samples. However, this difference between jugular and portal samples was more pronounced for PC levels and remained significant whatever the Child-Pugh score (Table 1).

D-dimers were significantly higher in portal samples (median = 3526 ng/ml vs. 2170 ng/ml in jugular blood, p = 0.008, Table 1). Prothrombin F1+2 levels were also significantly higher in portal samples (median = 835 pM vs. 252 pM in jugular blood, p < 0.0001). In addition, D-dimers and F1+2 levels were significantly correlated in both portal (r=0.61, p=0.002) and jugular veins (r=0.54, p= 0.0065).

Thrombin generation assay

TGA was first performed in all portal and jugular samples using relatively high concentrations of TF (5 pm) and PL (4 μM) (Procedure A), and the results obtained for the patient carrying the FII 20210A mutation were excluded from analysis.
The median values of peaks of IIa and ETP were significantly lower with the plasma from cirrhotic patients than those measured in controls whatever the procedure used (Table 2B). However, all parameters recorded (i.e. lag time, peak of IIa and ETP) were similar in jugular and portal samples.

When lower concentrations of TF and PL were used (Procedure B), lag times were markedly prolonged and peaks of IIa were reduced compared to values obtained with procedure A, while ETP values were only slightly lower (Table 2B). Median values of peaks of IIa were significantly lower in controls but without any relationship with the severity of cirrhosis. In contrast, ETP measured with this procedure was not significantly different compared to controls, and results obtained with jugular and portal plasma samples were also similar.

Thrombin generation was then measured in all samples after addition of TM. As expected, ETP values measured in jugular and portal blood were reduced compared to those obtained without TM, particularly when procedure B was applied (Table 2B). Importantly, ETP was always higher in cirrhotic plasma samples than in healthy control samples, but differences were significant only when procedure A was performed. Therefore, a resistance to TM was evidenced in both jugular and portal samples when TGA was performed without TM, particularly when procedure B was applied (Table 2B). Impor-

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<th>Table 1: Screening tests and specific assays. Median values and extreme values measured in all jugular and portal samples of cirrhotic patients are in bold, and those according to Child-Pugh scores are in italic. P-values (Wilcoxon signed rank test) measured after comparing results of portal and jugular samples are indicated by asterisks as follows: p &lt; 0.0001 = ****, p &lt; 0.001 = ***, p &lt; 0.01 = **, p &lt; 0.05 = *. P-values (Mann-Whitney U-test) measured after comparing the results obtained in jugular or portal plasma according to the Child-Pugh score only, are indicated by the letters a, b, c (Child B vs. Child A) and a’, b’, c’ (Child C vs. Child A) as follows: p &lt; 0.05 = ±, p &lt; 0.01 = ±± and p &lt; 0.001 = ±±±.</th>
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Discussion

In this study we investigated the coagulation balance of patients with liver cirrhosis requiring the implantation of TIPS. Thrombin generation was analyzed in the platelet-free plasma isolated after blood sampling in the jugular and portal veins of cirrhotic patients during the TIPS insertion procedure. The main aim of the study was to investigate whether a hypercoagulability could be evidenced in the splanchnic venous system compared to peripheral veins. This explains why we did not match the healthy subjects who were studied with our patients according to their age. Therefore, the selection of younger healthy controls likely reduced the level of significance of the results obtained in cirrhotic patients, since thrombin generation increases with age (23, 24).

However, our results obtained after performing TGA without TM showed lower peaks of thrombin in patients with liver disease compared to controls whatever the concentrations of reagents used.

ETP values were reduced particularly in patients with the most severe cirrhosis (Child-Pugh B and C) when the procedure B was applied. These results are in agreement with those of Tripodi et al. who showed impaired thrombin generation when testing the plasma from cirrhotic patients and in control plasma from healthy subjects. Procedure A (A) was performed with final concentrations of 5 pM tissue factor (TF) and 4 μM phospholipids (PL) in the absence or presence of 15 nM thrombomodulin (TM). Procedure B (B) was carried out with lower final concentrations of reagents (i.e. 1 pM TF and 1 μM PL ± 4 nM TM). No significant difference was found between jugular and portal samples whatever the procedure applied (non-parametric Mann-Whitney U test).

Table 2: TGA parameters measured in jugular and portal plasma samples from cirrhotic patients and in control plasma from healthy subjects. Procedure A (A) was performed with final concentrations of 5 pM tissue factor (TF) and 4 μM phospholipids (PL) in the absence or presence of 15 nM thrombomodulin (TM). Procedure B (B) was carried out with lower final concentrations of reagents (i.e. 1 pM TF and 1 μM PL ± 4 nM TM). No significant difference was found between jugular and portal samples whatever the procedure applied (non-parametric Mann-Whitney U test).
ma of cirrhotic patients without TM using similar and relatively low amounts of TF (1 pM) and PL (0.5 μM) (13). This reduced thrombin generation correlated well with FII levels that are of major importance in TGA, influencing both the peak of IIa and ETP (25, 26). FII levels were similar in portal and jugular plasma samples and this may explain why the results obtained with TGA did not support the existence of relative hypercoagulability in portal venous blood. In addition, fibrinogen, antithrombin, and free TFPI levels that are also important determinants of the amount of thrombin formed in TGA, particularly when performed with high concentrations of TF (24), were similar in the portal and jugular plasma of our patients. Moreover, TF levels were also comparable with higher values measured in severe cirrhosis. However, this TF did not appear to influence the TGA parameters that were measured, without any shortening of the lag-time and no spontaneous IIa generation within 15 min of monitoring when no exogenous TF was added to the plasma tested (data not shown). In contrast, when the concentration of TF added to jugular or portal plasma was increased from 1 pM to 5 pM, a shortened lag-time with an increase of thrombin peak was always recorded.

A TM resistance was evidenced in both jugular and portal venous plasma when TGA was performed after the addition of PC. Therefore, the plasma of cirrhotic patients generated more thrombin than that of healthy controls in the presence of TM, and the difference was greater for samples from patients with Child-Pugh scores B and C. PC levels were markedly decreased in these patients and this deficiency was more pronounced in portal blood. However, no difference in TM resistance could be evidenced between the portal and jugular samples. To evaluate the impact of low PC levels in this TM resistance, TGA was measured using procedure B before and after addition of purified PC in the portal and jugular plasma of two patients with severe cirrhosis, without and with TM tested at two concentrations (4 nM and 15 nM). Whatever the plasma tested (jugular or portal), the TM resistance remained unchanged with 15 nM of TM when no exogenous PC was added. In contrast, sensitivity to TM was restored when protein C levels were normalised with a reduction of ETP values higher than 50% in the presence of 15 nM of TM. However, no difference in TM sensitivity was found between portal and jugular plasma of the two patients tested when PC levels were nor-

Figure 1: TGA parameters measured in healthy controls (filled boxes) and in jugular (empty boxes) or portal samples (dotted boxes) from cirrhotic patients using procedure A without and with thrombomodulin (TM). The horizontal lines in the middle of box-and-whiskers plots show the median values (50th percentile). The top and bottom of boxes show the 25th and 75th percentiles. The whiskers define the 10th and 90th percentiles. Differences between patients and controls are indicated using asterisks as follows: p < 0.0001 = ***, p < 0.001 = ** and p <0.05= *. P-values below arrows indicate differences between groups of patients with different Child-Pugh scores (non-parametric Mann-Whitney U test).

Figure 2: Normalised TM sensitivity ratio (nTMSR) values measured in healthy controls (filled boxes) and in jugular (empty boxes) or portal samples (dotted boxes) from cirrhotic patients using procedure A. The horizontal lines in the middle of box-and-whiskers plots show the median values (50th percentile). The top and bottom of boxes show the 25th and 75th percentiles. The whiskers define the 10th and 90th percentiles. Differences between patients and controls are indicated using asterisks as follows: p < 0.0001 = ***, p < 0.001 = ** and p <0.05= *. P-values below arrows indicate differences between groups of patients with different Child-Pugh scores (non-parametric Mann-Whitney U test).
malised (see Supplementary data, available online at www.thrombosis-online.com). These results suggest that resistance to TM in cirrhosis is mainly related to the protein C deficiency even if some influence of FVIII level cannot be excluded. Indeed, nTMSR values well correlated with both FVIII and PC levels, but only when 1 pM TF was used to trigger the thrombin generation. Elevated FVIII levels are explained in liver cirrhosis by the increased hepatic biosynthesis of von Willebrand factor and decreased expression of low-density lipoprotein receptor related (LRP) proteins (27), and have also been associated with an increased risk of portal vein obstruction (28). In addition, enlarged portal veins appear to overgrow FVIII-producing sinusoid endothelial cells in cirrhosis (27), and this mechanism also possibly contributes to the higher FVIII levels in portal circulation. Variations in FVIII levels within the normal range do not significantly affect the ETP (24). But, a recent study showed that spiking plasma with purified FVIII to 200% or 400% resulted in significant increase in the ETP value after initiation of the coagulation cascade with 1 pM TF (29).

Interestingly, there are some similarities between the coagulation of neonates and that of patients with cirrhosis who both exhibit combined deficiencies of pro- and anticoagulant proteins modifying the haemostatic balance but without obvious reduction in thrombin generation when evaluated by TGA in the presence of TM (30). PC and PS are also decreased in neonates, with plasma levels comparable to those of cirrhotic patients, while FVIII concentration is relatively high and similar to that of healthy adults (31). These similar coagulation features in neonates and patients with liver impairment may therefore account for the resistance to TM evidenced in vitro by TGA in both categories of subjects. Tripodi et al. recently studied the thrombin generation in peripheral venous plasma samples of a large cohort of patients with chronic liver disease and also showed they were resistant to the effect of TM, with resulting hypercoagulability that was greater in severe cirrhosis (30).

The main pathogenic factor of PVT in cirrhosis is stasis of portal blood flow (32) but inherited or acquired modifications of co-

**Figure 3:** Correlation between normalised TM sensitivity ratio (nTMSR) values (1 pM TF, 1 μM PL, 4 nM TM) and PC (A) or FVIII levels (B) in jugular (empty circles, dotted lines) and portal blood (filled circles, continuous lines). Correlations were evaluated by the non-parametric Spearman rank test. The slopes of A and B were not statistically different (State Graphics Centurion Software®).

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agulation may also play a significant pathogenic role (33). However, the contribution of deficiencies in PC, PS and lower levels of protein C, but little was known about the coagulation balance in portal blood.

What does this paper add?
- This study supports the existence of a hypercoagulability in the portal plasma with higher FVIII, D-dimers and F 1+2 levels and lower PC levels compared to jugular plasma.
- However, a similar resistance to thrombomodulin was measured in jugular and portal plasma with the thrombin generation assay.

What is known about this topic?
- Liver cirrhosis is associated with a high risk of portal thrombosis favoured by venous stasis, but the mechanisms involved are incompletely defined.
- The hypercoagulability of peripheral plasma has recently been shown to result from increased levels of factor VIII and lower levels of protein C, but little was known about the coagulation balance in portal blood.

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