Enhanced expression of monocyte tissue factor in patients with liver cirrhosis

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Abstract

Background—Previous studies have shown that cirrhotic patients produce increased amounts of thrombin but the underlying mechanism is still unknown.

Aims—To analyse the relation between the rate of thrombin generation and monocyte expression of tissue factor (TF) in cirrhosis.

Patients—Thirty three cirrhotic patients classified as having low (n=7), moderate (n=17), or severe (n=9) liver failure according to Child-Pugh criteria.

Methods—Prothrombin fragment F1+2, monocyte TF activity and antigen, and endotoxaemia were measured in all patients. Polymerase chain reaction (PCR) analysis of TF mRNA was performed in monocytes of five cirrhotic patients.

Results—Prothrombin fragment F1+2 was higher in cirrhotic patients than in controls (p<0.0001). Monocytes from cirrhotic patients had higher TF activity and antigen than those from controls (p<0.0001) with a progressive increase from low to severe liver failure. Monocyte expression of TF was significantly correlated with plasma levels of F1+2 (TF activity: r=0.98, p<0.0001; TF antigen: r=0.95, p<0.0001) and with endotoxaemia (TF activity: r=0.94, p<0.0001; TF antigen: r=0.91, p<0.0001). PCR analysis of TF mRNA showed TF expression only in three patients with endotoxaemia (more than 15 pg/ml).

Conclusions—Cirrhotic patients have enhanced expression of TF which could be responsible for clotting activation, suggesting that endotoxaemia might play a pivotal role.

Keywords: prothrombin fragment F1+2; tissue factor; monocyte

Among the complex haemostatic disturbances occurring in liver cirrhosis, hyperfibrinolysis is considered one of the most frequent disorders observed in patients with the decompensated state.1 However, it is still debated whether hyperfibrinolysis is primary or secondary to clotting activation.2 Recently, we provided evidence that in patients with liver cirrhosis hyperfibrinolysis is secondary to an ongoing prothrombotic state which was documented by elevated circulating levels of the prothrombin fragment F1+2, a marker of in vivo thrombin generation.3 Clotting activation was particularly evident in patients with moderate to severe liver failure, suggesting a role for liver dysfunction in accelerating the thrombin generation rate.

Tissue factor (TF) is a membrane bound glycoprotein which is induced or expressed by circulating monocytes by means of specific inflammatory mediators and triggers the extrinsic pathway of the clotting system.4 Calmus and Robert5 found increased procoagulant activity in monocytes of cirrhotic patients but they did not investigate whether this was a result of enhanced expression of TF. The aim of the present study was to analyse the relation between clotting activation and monocyte expression of TF in cirrhotic patients with different degrees of liver failure.

Materials and methods

SUBJECTS

A total of 33 patients (23 males, 10 females; aged 39–72 years) with liver cirrhosis and 14 healthy volunteers (6 males, 8 females; aged 27–79 years) was studied. In all patients the diagnosis of cirrhosis was supported by liver needle biopsy. In patients with severe liver failure and coagulopathy, diagnosis was based on liver biopsy performed during a previous hospital stay. Patients were excluded if they had: hepatocarcinoma, diagnosed by the combination of hepatic ultrasound and/or computed tomography, and serum a fetoprotein; spontaneous bacterial peritonitis or other infectious diseases, diagnosed by clinical (fever and/or abdominal pain) and laboratory (ascitic and blood culture, polymorphonuclear count in ascitic fluid) indices; or cholestatic liver disease.

All patients gave informed consent to inclusion in the study. The standard treatment consisted of spironolactone, furosemide, ethacrinic acid, albumin, and lactulose. Patients were not given non-absorbable antibiotics or any other type of antibiotic in the previous 30 days. Patients were excluded from the study if there was an immediate need for blood or plasma. Patients included in the study gave a complete clinical history with particular reference to previous bleeding; nine patients had gastrointestinal bleeding at least three months before enrolment in the study. All patients underwent a complete physical examination with the purpose of scoring liver failure. Degree of liver failure was defined as low (class A), moderate (class B), or severe (class C) according to the Child-Pugh criteria, including clinical (ascites, encephalopathy) and laboratory (albumin, bilirubin, prothrombin time) parameters. Seven (21%) patients were assigned to class A, 17 (51%) to class B, and nine (28%) to class C.
Among the 33 patients, 11 (33%) had serological markers for hepatitis B virus (HBV), 14 (42%) had markers for hepatitis C virus (HCV), and eight (24%) had a history of alcoholism. This study was approved by the Internal Medicine Review Board of the Institution.

STUDY DESIGN
A cross-sectional study including the measurement of monocyte TF expression, plasma levels of F1+2, and endotoxemia was carried out in cirrhotic patients and controls.

In a second study TF mRNA was measured in monocytes taken from cirrhotic patients who were selected if they had normal leucocyte count and gave informed consent. This selection was necessary due to the large amount (80 ml) of blood required.

BLOOD COAGULATION STUDY
Between 8.00 and 9.00 am, a blood sample (nine parts) was obtained from patients and healthy volunteers; it was mixed with 3.8% sodium citrate (one part) and treated for study of the clotting system, as reported below. Patients had fasted for at least 12 hours and had not taken any drug known to interfere with the clotting system, as reported below.

Monocyte TF expression, plasma TF-VII complexes and is designed such that there is no interference from other coagulation factors or inhibitors of procoagulant activity.

ENDOTOXIN ASSAY
The measurement of endotoxemia was performed according to a commercially chromogenic substrate test (Enzygnost F1+2, Behringwerke, Marburg, Germany; reference value 0.6 (0.2) nM, range 0.3–1.2). Endotoxin assay performed using a commercial kit (Imubind Tissue Factor ELISA Kit, American Diagnostica Inc., Greenwich, Connecticut). The lower detection limit is approximately 10 pg/ml. The assay recognises TF-apo, TF, and TF-VII complexes and is designed such that there is no interference from other coagulation factors or inhibitors of procoagulant activity.

PCR ANALYSIS OF TF mRNA
Oligonucleotides F1 (sense bp 178–198) and R1 (antisense bp 495–515) from the coding sequence of human TF, and GF1 (sense bp 64–86) and GR1 (antisense bp 581–603) from the coding sequence of human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used to amplify TF mRNA. The primers were designed using the Primer 3 software (http://frodo.wi.mit.edu/primer3). The PCR conditions were as follows: initial denaturation at 94 °C for 3 min, followed by 30 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min, and a final extension at 72 °C for 5 min. The PCR products were separated on a 1% agarose gel and visualized by ethidium bromide staining.

Tissue factor (TF) mRNA expression was evaluated by quantitative real-time PCR (LightCycler, Roche Diagnostics). The relative expression levels were calculated using the ΔΔCt method and normalised to the expression of GAPDH as an internal reference.

Saline (PBS) was used as a control condition. The data were analysed using one-way ANOVA followed by Tukey’s multiple comparison test. A p value <0.05 was considered statistically significant.
Table 1  Clinical and laboratory characteristics of patients with liver cirrhosis

<table>
<thead>
<tr>
<th>Variable</th>
<th>Controls (n=14)</th>
<th>Grade A cirrhosis (n=17)</th>
<th>p Value (A v B)</th>
<th>Grade B cirrhosis (n=17)</th>
<th>p Value (B v C)</th>
<th>Grade C cirrhosis (n=17)</th>
<th>p Value (A v C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean (SD) age (y)</td>
<td>52 (16)</td>
<td>57 (11)</td>
<td>NS</td>
<td>62 (8)</td>
<td>NS</td>
<td>59 (17)</td>
<td>NS</td>
</tr>
<tr>
<td>No of men (%)</td>
<td>6 (43)</td>
<td>5 (71)</td>
<td>NS</td>
<td>11 (65)</td>
<td>NS</td>
<td>7 (78)</td>
<td>NS</td>
</tr>
<tr>
<td>Unstimulated TF activity (U/2×10^5 monocytes)</td>
<td>H=19.9, p&lt;0.001</td>
<td>0 (&lt;1.0–9)</td>
<td>NS</td>
<td>11.0 (&lt;1.0–15)</td>
<td>&lt;0.05</td>
<td>41 (29–55)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>LPS (200 pg/ml) stimulated TF activity (U/2×10^5 monocytes)</td>
<td>H=12.5, p&lt;0.002</td>
<td>9 (4–22)</td>
<td>NS</td>
<td>30 (13–50)</td>
<td>&lt;0.05</td>
<td>42 (33–64)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Unstimulated TF antigen (pg/2×10^5 monocytes)</td>
<td>H=15.0, p&lt;0.001</td>
<td>11 (&lt;10–15)</td>
<td>24 (10–79)</td>
<td>&lt;0.05</td>
<td>80 (54–142.6)</td>
<td>&lt;0.05</td>
<td>80 (54–142.6)</td>
</tr>
<tr>
<td>LPS (200 pg/ml) stimulated TF antigen (pg/2×10^5 monocytes)</td>
<td>H=18.5, p&lt;0.001</td>
<td>16 (10–40)</td>
<td>62 (23.6–98.9)</td>
<td>&lt;0.05</td>
<td>81.1 (62.5–144)</td>
<td>&lt;0.05</td>
<td>81.1 (62.5–144)</td>
</tr>
<tr>
<td>F1+2 (nM) (95% CI)</td>
<td>0.6 (0.3)</td>
<td>0.9 (0.3)</td>
<td>&lt;0.05</td>
<td>1.5 (0.6)</td>
<td>&lt;0.05</td>
<td>3.4 (0.7)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Endotoxaemia (pg/ml) H=22.6, p&lt;0.0001</td>
<td>4.1 (9–9.6)</td>
<td>13.4 (4.4–17.5)</td>
<td>&lt;0.05</td>
<td>20.5 (10.5–36.5)</td>
<td>&lt;0.05</td>
<td>72 (43–137.5)</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

H, Kruskal-Wallis test among Child-Pugh classes; TF, tissue factor; F, analysis of variance among Child-Pugh classes; F1+2, human prothrombin fragment F1+2.

Phosphate dehydrogenase (GAPDH) were synthesised. To obtain the first cDNA strand 1 µg of total RNA from mononuclear leucocytes was reverse transcribed using random hexamers and Moloney murine leukaemia virus reverse transcriptase. Polymerase chain reaction (PCR) was performed with 5 µl of cDNA in 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM MgCl2, 0.4 µg of each appropriate sense and antisense primer, 200 mM of each dNTP, and 2.5 units of Taq polymerase. The amplification conditions were 94°C for one minute, 60°C for one minute, and 72°C for one minute to obtain a product of 528 bp from GAPDH mRNA and 317 bp from TF mRNA. The PCR was performed in the exponential phase (cycle 23 for GAPDH or 32 for TF), as assessed in previous experiments, in which at various cycles (21, 23, 25, 28, 32, 35) the PCR products were tested (data not shown). An 8 µl aliquot of the reactions was analysed on 1% agarose gel stained with ethidium bromide and subsequently by Southern blotting, for a semi-quantitative analysis of PCR products for TF. The relative intensity of the bands visualised by autoradiography was measured by laser densitometry.

STATISTICAL ANALYSIS

Statistical analysis was performed by χ² statistics or Fisher’s exact test (if n<5) for independence and by appropriate analysis of variance and/or t test. When necessary, log transformation was used to normalise the data, or appropriate non-parametric tests were employed. The linear regression test or Spearman rank correlation test was used to study the different correlations. Data are presented as mean (SD) and 95% confidence intervals or as median (range). Only two tailed probabilities were used for testing statistical significance. Probability values less than 0.05 were regarded as statistically significant. All calculations were made with the computer program StatView II (Abacus Concepts, Berkeley, California).

Results

According to our previous work, patients with liver cirrhosis had an increased rate of thrombin generation compared with controls (1.2–1.8) versus 0.9 (0.3) nM, p<0.0001). F1+2 plasma values progressively increased from low to severe liver failure (p<0.0001) (table 1).

In the absence of endotoxin, control monocytes generated low TF activity, while monocytes from cirrhotic patients had elevated levels (median (range): 11.0 (<1.0–9) versus 20.5 (10.5–36.5) pg/10^5 monocytes, p<0.001). When patients with different degrees of liver failure were compared, there was a progressive increase in TF activity from A to C class (p<0.0001) (table 1, fig 1). The relation between TF activity and degree of liver failure was also observed with endotoxin stimulated
monocytes (table 1). When TF antigen was measured in lysed cells, TF expressed by untreated monocytes was significantly higher in cirrhotic patients than in controls (median (range): 54 (10–142.6) versus 11.5 (<10–15) pg/2 × 10^5 monocytes, p<0.0001). The values of TF antigen from unstimulated monocytes progressively increased from A to C class (p<0.001) (table 1, fig 2). Similar findings were observed with endotoxin stimulated monocytes (table 1). Both TF activity (r=0.98, p<0.0001) and antigen (r=0.95, p<0.0001) were significantly correlated with F1+2 (figs 3 and 4).

Cirrhotic patients had higher values of endotoxaemia than controls (median (range): 20.9 (4.4–137.5) versus 4.1 (0–9.6) pg/ml, p<0.0001) with a progressive increase from low to severe liver failure (p<0.0001) (table 1). A strong correlation was observed between F1+2 and endotoxaemia (r=0.94, p<0.0001) (table 1). A strong correlation was observed between endotoxaemia and TF activity (r=0.94, p<0.0001) and TF activity and antigen (r=0.91, p<0.0001).

Nine (27%) of 33 patients examined had a clinical history of gastrointestinal bleeding. Similar values of F1+2, endotoxaemia, and TF activity and antigen were observed in patients who bled and those who did not (not shown).

To determine the steady state levels of TF mRNA, we reverse transcribed RNA from cells obtained from five different patients and amplified it using PCR. To evaluate TF mRNA levels, a Southern blot analysis of the PCR product during amplification was performed. Table 2 shows clinical and laboratory characteristics of these patients. All patients misused alcohol; one was of A class, two of B class, and two of C class. Endotoxaemia was close to the upper limits of control values in patients 1 and 2 and elevated in patients 3, 4, and 5. In these patients TF activity and antigen were measured in PBMC lysate, immediately after cell separation. In patients with almost normal endotoxaemia spontaneous TF activity was absent and the expression of TF antigen was close to the lower detection limit of the ELISA assay; patients with elevated endotoxaemia showed elevated TF activity and antigen (table 2). The PCR products for TF behaved accordingly. Those from cells obtained from patients 1 and 2 were not visible, indicating that the expression of TF mRNA is negligible (fig 5).

The level of TF mRNA of cells from patients 3, 4, and 5 was clearly evident and associated with elevated TF activity and antigen.

Discussion

Activation of the clotting system seems to be the mechanism leading to hyperfibrinolysis as the presence of systemic signs of hyperfibrinolysis (elevated plasma values of D-dimer), is closely related to an enhanced rate of thrombin generation. Furthermore, the enhanced rate of thrombin generation was significantly associated with tissue plasminogen activator plasma levels, further suggesting that hyperfibrinolysis is secondary to clotting activation.

The results of the present study further reinforce the concept that in cirrhosis there is an ongoing prothrombotic state as we showed that cirrhotic patients, particularly those with moderate to severe liver failure, have an enhanced expression of TF activity and antigen. These

<table>
<thead>
<tr>
<th>Patient no</th>
<th>Child-Pugh class</th>
<th>F1+2 (nM)</th>
<th>Endotoxin (pg/ml)</th>
<th>Unstimulated TF activity (U/2 × 10^5 monocytes)</th>
<th>Tissue factor antigen (pg/2 × 10^5 monocytes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>B</td>
<td>0.6</td>
<td>15</td>
<td>&lt;1.0</td>
<td>11</td>
</tr>
<tr>
<td>2</td>
<td>A</td>
<td>0.7</td>
<td>9.8</td>
<td>&lt;1.0</td>
<td>12.5</td>
</tr>
<tr>
<td>3</td>
<td>B</td>
<td>2.2</td>
<td>43</td>
<td>34</td>
<td>70</td>
</tr>
<tr>
<td>4</td>
<td>C</td>
<td>3.35</td>
<td>72</td>
<td>41</td>
<td>106.2</td>
</tr>
<tr>
<td>5</td>
<td>C</td>
<td>3.0</td>
<td>62</td>
<td>29</td>
<td>54</td>
</tr>
</tbody>
</table>
data are relevant to the understanding of the pathophysiology of clotting disturbances in cirrhosis as the overexpression of monocyte TF may represent an important stimulus for the clotting system.

Consistent with this suggestion is the strong correlation between monocyte TF expression and prothrombin F1+2 circulating levels. It is interesting to note that, compared with controls, enhanced expression of TF was found not only in lipopolysaccharide (LPS) stimulated monocytes but also in monocytes not exposed to LPS. This could indicate that in cirrhosis monocytes are activated in vivo. In accordance with this suggestion, TF mRNA could be detected in some patients with moderate to severe liver failure, suggesting that in cirrhosis there is some factor which contributes to monocyte activation. To explore this issue, we focused our attention on endotoxaemia which is elevated in cirrhosis as a consequence of impaired liver clearance and is an important trigger of clotting activation.

We found a significant correlation between endotoxaemia and F1+2 plasma levels, therefore endotoxaemia may represent an important stimulus for monocyte activation in cirrhosis. Thus, in a range of concentrations (50–200 pg/ml) close to that found in cirrhosis, LPS enhances monocyte expression of TF. Interestingly, in our cohort of cirrhotic patients with moderate to severe liver failure, endotoxaemia was on average 50 pg/ml; this concentration may therefore be an adequate stimulus for monocyte activation. In accordance with this suggestion we found a significant correlation between monocyte expression of TF and endotoxaemia; furthermore, TF mRNA was not detected in patients with almost normal endotoxaemia while patients with endotoxaemia of more than 15 pg/ml had detectable TF mRNA. Until now, TF expression by circulating monocytes has been observed in experimental and clinical models such as septic shock and malignant disease. Cirrhosis could represent another model in which low grade endotoxaemia induces monocyte TF expression, but further study is necessary to prove definitely a cause and effect relation.

We cannot exclude the fact that other cells may contribute to activation of the clotting system as it has been shown that in cirrhosis, endotoxaemia may also induce endothelial perturbation. This suggestion should be investigated as activation of the clotting system could also result from endotoxin stimulation of endothelial cells, which are known to express TF on appropriate stimulation.

In conclusion, this study shows that cirrhotic patients have enhanced monocyte TF expression, which may be responsible for systemic clotting activation, and suggests that endotoxaemia might play a pivotal role.

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