Enhanced expression of monocyte tissue factor in patients with liver cirrhosis

M Saliola, R Lorenzet, D Ferro, S Basili, C Caroselli, A Di Santo, M Sallese, F Violi

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<0.001) 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.

**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 biopsy. Patients were excluded if they had: hepatocarcinoma, diagnosed by the combination of hepatic ultrasound and/or computed tomography, and serum α 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) indexes; 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 endotoxaemia 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 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 coagulation or the fibrinolytic system in the previous 15 days. Citrated blood samples were immediately centrifuged at 4°C for 20 minutes at 2000 g, and the supernatant was stored at −80°C until use.

Plasma levels of human prothrombin fragment F1+2 were assayed by an enzyme immunoassay based on the sandwich principle (Enzygnost F1+2, Behringwerke, Marburg, Germany; reference value 0.6 (0.2) nM, range 0.3–1.2). Intra-assay and interassay coefficients of variation were 8% and 9% respectively.

ENDOTOXIN ASSAY
The measurement of endotoxaemia was performed according to a commercially chromogenic substrate test (Kabi-Diagnostica, Stockholm, Sweden) as previously described. Blood sampling and endotoxin assay were performed in a laminar flow bank (Biohazard VBH-48 MP Steril, Milan, Italy), and all laboratory material was sterile, showing a linear relation between endotoxin concentration and absorbance over the 0–200 pg/ml range. All sample assays were performed in duplicate. In addition, two blank values were prepared for each test sample by adding endotoxin free water instead of the same volume of diluted plasma or Limulus amoebocyte lysate (LAL) respectively. This procedure ensures that an aseptic technique has been used. The day to day coefficient of variation was 11% (reference value 4.5 (2.6) pg/ml).

ISOLATION AND INCUBATION OF BLOOD MONONUCLEAR CELLS
Peripheral blood mononuclear cells (PBMCs) were isolated from heparinised venous blood using an aseptic technique. Platelets were removed using two step centrifugation: once at 140 g and twice at 100 g in phosphate buffered saline (PBS) at room temperature for 10 minutes. PBMCs were isolated by centrifugation on lymphoprep (Nyegaard, Oslo, Norway) at 1200 g for 20 minutes at 20°C. Monocytes, identified by May Grunwald Giemsa staining, comprised 16–22% (mean 19%). Monocytes (adherent cells) were obtained by incubation of the PBMCs for 90 minutes at 37°C in a humidified atmosphere of 5% CO2 in air in Petri dishes containing RPMI 1640, supplemented with 2 mM glutamine; lymphocytes (non-adherent cells) were removed by aspiration with a Pasteur pipette and washing of the dishes with warm media. The purified monocyte preparation contained 85–95% monocytes. After isolation cells were washed twice in PBS and incubated at 2×10⁷ cells/ml in RPMI 1640 at 37°C, 5% CO2, for six hours. Cultures were performed either without stimuli or in the presence of lipopolysaccharide (LPS; Escherichia coli OB11:B4, Sigma, St Louis, Missouri, USA) at a final concentration of 200 pg/ml. At the end of the incubation period, cells and media were separated by centrifugation (2000 g for 15 minutes). The cells were washed with Tris-NaCl buffer (0.1 mmol/l NaCl, 0.1% bovine serum albumin, pH 7.4), then lysed in the same buffer by adding 15 mmol/l n-octyl-D-glycopyranoside at 37°C for 30 minutes. Cell count and trypan blue exclusion were performed on cell suspensions after washing.

TISSUE FACTOR ASSAY
TF activity was determined in the cell lysate, by measuring monocyte procoagulant activity with a one stage clotting assay. In five patients TF activity was measured in PBMC lysate, within one hour of blood withdrawal. Briefly, 100 µl aliquots of cell lysate were added to 100 µl of normal pooled citrated plasma; after 150 seconds incubation at 37°C, 100 µl of 0.025 mM CaCl2 was added and clotting time recorded. All samples were tested in duplicate. Clotting times were converted to arbitrary TF units (U)/2×10⁷ monocytes using logarithmic plots of clotting time versus dilution of a standard TF solution obtained using commercial thromboplastin (Dade). Undiluted thromboplastin was assigned a value of 1000 TF units, corresponding to a clotting time of 14 seconds. This procoagulant activity was not seen with plasma deficient in factors VII, X, or V.

The enzyme linked immunosorbent assay (ELISA) for measuring TF antigen in cell lysate was 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 recognise 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
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 (n=7)</th>
<th>p Value</th>
<th>Grade B (n=17)</th>
<th>p Value</th>
<th>Grade C (n=8)</th>
<th>p Value</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>
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<tr>
<td>Unstimulated TF activity (U/2 × 10⁵ monocytes)</td>
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<tr>
<td>H=19.9, p&lt;0.0001</td>
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<tr>
<td>LPS (200 pg/ml) stimulated TF activity (U/2 × 10⁵ monocytes) H=12.5, p&lt;0.002</td>
<td>0 (&lt;1.0–9)</td>
<td>3.0 (&lt;1.0–15)</td>
<td>NS</td>
<td>11.0 (&lt;1.0–32)</td>
<td>&lt;0.05</td>
<td>41 (29–55)</td>
<td>&lt;0.05</td>
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<tr>
<td>Unstimulated TF antigen (pg/2 × 10² monocytes)</td>
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<tr>
<td>H=17.0, p&lt;0.0001</td>
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<tr>
<td>LPS (200 pg/ml) stimulated TF antigen (pg/2 × 10² monocytes) H=15.8, p&lt;0.001</td>
<td>11 (&lt;10–15)</td>
<td>22 (6–45)</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>
</tbody>
</table>

Tissue factor activity (U/2 × 10⁵ monocytes) versus liver failure (Table 1).

Figure 1  Tissue factor activity in unstimulated monocytes from controls and from cirrhotic patients with low (grade A), moderate (grade B), and severe (grade C) liver failure.

Figure 2  Tissue factor antigen in unstimulated monocytes from controls and from patients with low (grade A), moderate (grade B), and severe (grade C) liver failure.

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 nM 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 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 (1.9 (1.1) versus 0.6 (0.2) 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): <1.0 (<1.0–9) versus 20 (1.9–158) U/2×10⁷ 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
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); also, a strong correlation was observed between endotoxaemia and TF activity (r=0.94, p<0.0001) 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 2 Clinical and laboratory characteristics of five cirrhotic patients with low or raised endotoxaemia**

<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 enhanced 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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