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.

(Gut 1998;43:428-432)

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

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.⁴⁻⁵ Calmus and Robert⁶ 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 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.

Institute of Clinical Medicine I, University of Rome "La Sapienza"
M Saliola
D Ferro
S Basili
C Caroselli
F Violi

Antonio Taticchi Unit for Atherosclerosis and Thrombosis
R Lorenzet
A Di Santo

Laboratory of Molecular Biology and Pharmacology of Receptors, Istituto di Ricerche Farmacologiche Mario Negri, S Maria Imbaro, Italy
M Sallese

Correspondence to: Professor F Violi, Clinica Medica, Policlinico Umberto I, Viale del Policlinico, 00185, Rome, Italy.

Accepted for publication 18 February 1998

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

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).³ 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.³ 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.⁶ 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.⁷ The purified monocyte preparation contained 85–95% monocytes. After isolation cells were washed twice in PBS and incubated at 2×10⁵ 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.⁸ 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)/2×10⁵ 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 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

Figure 3 Correlation between tissue factor activity in unstimulated monocytes and prothrombotic fragment F1+2 in cirrhotic patients.

Figure 4 Correlation between tissue factor activity in unstimulated monocytes and prothrombin fragment F1+2 in cirrhotic patients.

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/2×10⁵ 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

Figure 5 Tissue factor mRNA levels in monocytes from cirrhotic patients. Patient numbers (1–5) correspond to those reported in table 2.

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.³ 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.³

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

Patient no	Child-Pugh class	F1+2 (nM)	Endotoxin (pg/ml)	Unstimulated TF activity (U/2 × 10 ⁵ monocytes)	Tissue factor antigen (pg/2 × 10 ⁵ monocytes)
1	B	0.6	15	<1.0	11
2	A	0.7	9.8	<1.0	12.5
3	B	2.2	43	34	70
4	C	3.35	72	41	106.2
5	C	3.0	62	29	54

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 clearance¹¹ and is an important trigger of clotting activation.³ We found a significant correlation between endotoxaemia and F1+2 plasma levels, therefore endotoxaemia may represent an important stimulus for monocyte activation in cirrhosis.⁵ Thus, in a range of concentrations (50–200 pg/ml) close to that found in cirrhosis, LPS enhances monocyte expression of TF.¹² 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.^{13–16} 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.¹⁷ 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.^{18–20}

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.

This work was in part supported by the Italian National Research Council (Convenzione CNR-Consorzio Mario Negri Sud).

- 1 Violi F, Ferro D, Basili S, et al. Hyperfibrinolysis increases the risk of gastrointestinal hemorrhage in patients with advanced cirrhosis. *Hepatology* 1992;15:672–6.
- 2 Violi F, Basili S, Ferro D, et al, and CALC group. Association between high values of D-dimer and tissue-plasminogen activator activity and first gastrointestinal bleeding in cirrhotic patients. *Thromb Haemost* 1996;76:177–83.
- 3 Violi F, Ferro D, Basili S, et al. Association between low-grade disseminated intravascular coagulation and endotoxaemia in patients with liver cirrhosis. *Gastroenterology* 1995;109:531–9.
- 4 Edginton TS, Mackman N, Brand K, et al. The structural biology of expression and function of tissue factor. *Thromb Haemost* 1991;66:67–79.
- 5 Nemerson Y. Tissue factor and hemostasis. *Blood* 1988;71:1–8.
- 6 Calmus Y, Robert A. Increased procoagulant activity in peripheral blood mononuclear cells from patients with liver cirrhosis. *Thromb Res* 1992;68:103–8.
- 7 Kornberg A, Blank M, Kaufman S, et al. Induction of tissue factor-like activity in monocytes by anti-cardiolipin antibodies. *J Immunol* 1994;153:1328–32.
- 8 Meszaros K, Aberle S, Dedrick R, et al. Monocytes tissue factor induction by lipopolysaccharide (LPS): dependence on LPS-binding protein and CD14, and inhibition by a recombinant fragment of bactericidal/permeability-increasing protein. *Blood* 1994;83:2516–25.
- 9 Celi A, Pellegrini G, Lorenzet R, et al. P-selectin induces the expression of tissue factor on monocytes. *Proc Natl Acad Sci USA* 1994;91:8767–71.
- 10 Armitage P, Berry G. *Statistical methods in medical research*, 2nd edn. Oxford: Blackwell Scientific Publications, 1990: 200–15.
- 11 Liehr H. Endotoxins and the pathogenesis of hepatic and gastrointestinal diseases. *Adv Int Med Ped* 1982;48:117–93.
- 12 Osterud B, Bjorklid E. The production and availability of tissue thromboplastin in cellular populations of whole blood exposed to various concentrations of endotoxin. *Scand J Haematol* 1982;29:175–84.
- 13 Osterud B, Flaegstad T. Increased tissue thromboplastin activity in monocytes of patients with meningococcal infection: related to an unfavourable prognosis. *Thromb Haemost* 1983;49:5–7.
- 14 Warr TA, Rao LVM, Rapaport SI. Disseminated intravascular coagulation in rabbits induced by administration of endotoxin or tissue factor. Effect of anti-tissue factor antibodies and measurement of plasma extrinsic pathway inhibitory activity. *Blood* 1990;75:1481–9.
- 15 Taylor FB Jr, Chang A, Ruf W, et al. Lethal E. coli septic shock is prevented by blocking tissue factor with monoclonal antibody. *Circ Shock* 1991;33:127–34.
- 16 Semeraro N, Colucci M. Tissue factor in health and disease. *Thromb Haemost* 1997;78:759–64.
- 17 Ferro D, Quintarelli C, Lattuada A, et al. High plasma levels of von Willebrand factor as a marker of endothelial perturbation in cirrhosis: relationship to endotoxaemia. *Hepatology* 1996;23:1377–83.
- 18 Colucci M, Balconi G, Lorenzet R, et al. Cultured human endothelial cells generate tissue factor in response to endotoxin. *J Clin Invest* 1983;71:1893–6.
- 19 Bevilacqua MP, Pober JS, Majesau GR, et al. Recombinant tumor necrosis factor induces procoagulant activity in cultured human vascular endothelium: characterization and comparison with the actions of interleukin 1. *Proc Natl Acad Sci USA* 1986;83:4533–7.
- 20 Moldow CF, Bach RR, Staskus K, et al. Induction of endothelial tissue factor by endotoxin and its precursors. *Thromb Haemost* 1993;70:702–6.