

HEMOSTASIS, THROMBOSIS, AND VASCULAR BIOLOGY

Vitamin E Reduces Monocyte Tissue Factor Expression in Cirrhotic Patients

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Clotting activation may occur in liver cirrhosis, but the pathophysiological mechanism has not been fully elucidated. Because a previous study demonstrated that lipid peroxidation is increased in cirrhosis, we analyzed whether there is a relationship between lipid peroxidation and clotting activation. Thirty cirrhotic patients (19 men and 11 women; age, 34 to 79 years) and 30 controls matched for sex and age were investigated. In all subjects, monocyte expression of tissue factor (TF) antigen and activity; plasma levels of prothrombin fragment 1+2 (F1+2), a marker of thrombin generation; and urinary excretion of Isoprostane-F_{2α}-III, a marker of lipid peroxidation, were measured. Furthermore, the above-reported variables were re-evaluated after 30 days of treatment with standard therapy (n = 5) or standard therapy plus 300 mg vitamin E twice daily (n = 9). In addition, we analyzed in vitro if vitamin E (50 μmol/L) influenced monocyte TF expression and F1+2 generation. Cirrhotic

AMONG THE COMPLEX hemostatic disturbance occurring in liver cirrhosis (LC), hyperfibrinolysis is considered one of the most important factors that might favor hemorrhage.¹⁻⁴ Hence, the elucidation of the mechanism leading to hyperfibrinolysis would help us to explore new therapeutic strategies to prevent bleeding. Recently, we provided evidence that in LC patients hyperfibrinolysis is secondary to an ongoing prothrombotic state, which was documented by elevated circulating levels of the prothrombin fragment 1+2 (F1+2), a marker of in vivo thrombin generation.⁵ Clotting activation was particularly evident in patients with moderate-severe liver failure, suggesting a role for liver dysfunction in accelerating thrombin generation rate. Cross-sectional as well as interventional study provided evidence that endotoxemia, which is elevated in cirrhosis as a consequence of impaired hepatic clearance,⁶ might play a pivotal role. Thus, in cirrhotic patients receiving oral nonabsorbable antibiotics, the decrease of endotoxemia was closely associated with a significant reduction of F1+2 plasma levels.⁵

The relationship between clotting activation and endotoxemia has been recently investigated by measuring the monocyte expression of tissue factor (TF). We found that cirrhotic patients have an enhanced monocyte TF expression that correlated significantly with endotoxemia.⁷ Even if a cause-effect relationship between endotoxemia and TF expression was not documented in cirrhotic patients, several clinical and experimental models demonstrated that endotoxemia enhances the monocyte TF expression.⁸⁻¹⁰ However, an important still-open issue was to analyze the mechanism allowing endotoxemia to enhance TF expression and, in turn, clotting activation. Recent studies demonstrated that bacterial lipopolysaccharide (LPS) enhances monocyte formation of oxidant species,^{11,12} suggesting that oxygen free radicals, in particular peroxides, may represent an important intracellular mechanism by which LPS activates monocytes. Consistently, several antioxidant agents have been proved to decrease in vitro LPS-mediated monocyte TF expression.^{13,14} We have recently showed that lipid peroxidation is enhanced in cirrhosis and that endotoxemia could play an

important role.¹⁵ Isoprostane-F_{2α}-III was used as a marker of lipid peroxidation, because it is elevated in clinical settings associated with in vivo oxidant stress^{16,17} and is generated during low density lipoprotein (LDL) oxidation in vitro in coincidence with lipid peroxides formation.^{18,19} Therefore, we performed a cross-sectional study as well as an interventional study, using vitamin E as an antioxidant agent, to investigate whether a relationship between lipid peroxidation and clotting activation does exist in patients with cirrhosis.

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MATERIALS AND METHODS

Subjects

Thirty consecutive patients with hepatic cirrhosis (11 women and 19 men; age, 60 ± 11 years; age range, 34 to 79; 15 current smokers) and 30 healthy volunteers (18 men and 12 women; age, 55 ± 9 years; age range, 40 to 75 years; 10 current smokers) were studied. The diagnosis of cirrhosis was established by liver needle biopsy in all patients. All patients showed normal renal function. Patients were excluded from consideration if they had: (1) hepatocarcinoma, diagnosed by the combination of hepatic ultrasound and/or computed tomography together with elevated serum levels of α-fetoprotein; (2) spontaneous bacterial peritonitis or other infectious diseases, as indicated by clinical signs (fever and/or abdominal pain) and attendant (ascitic and blood culture, polymorphonuclear count in ascitic fluid) indexes; or (3) cholestatic liver disease.

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The Internal Medicine Review Board of the University Hospital of Rome approved the study. All subjects gave informed consent to their inclusion in the study. All abstained from nonabsorbable antibiotics and vitamin supplements for 30 days before the study. In case of immediate need for blood or plasma, patients were excluded from the study. The degree of liver failure was scored as mild (class A; $n = 6$), moderate (class B; $n = 20$), or severe (class C; $n = 4$) according to the Child-Pugh's criteria, based on clinical (ascites, encephalopathy) and laboratory (albumin, bilirubin, prothrombin time) parameters, as previously described.⁵ The etiology of cirrhosis was post-hepatitis B in 10 (33%) patients, post-hepatitis C in 18 (60%) patients, and postalcoholic in 2 (7%) patients.

Design of the Studies

In vivo study. In a first study, a cross-sectional analysis including the measurement of monocyte TF expression, plasma levels of prothrombin F1+2, endotoxemia, and urinary excretion of Isoprostane- $F_{2\alpha}$ -III was performed in cirrhotic patients and controls.

A second study was designed to explore the effect of vitamin E supplementation on monocyte TF expression and systemic clotting activation. To this purpose, 14 of 30 cirrhotic patients (8 women and 6 men; age, 42 to 75 years; 1 of A, 9 of B, and 4 of C class) who gave informed consent to participate also in this study received 300 mg vitamin E twice daily plus standard treatment ($n = 9$) or continued standard treatment ($n = 5$) for 30 days. Standard treatment consisted of spironolactone, furosemide or ethacrinic acid, albumin, and lactulose. The two groups were homogeneous for sex, age, and degree of liver failure. Monocyte TF expression, F1+2 plasma levels, and urinary excretion of Isoprostane- $F_{2\alpha}$ -III were evaluated before and at the end of the treatment period.

In vitro study. The study was performed to analyze the effect of vitamin E coinubation on LPS-stimulated monocyte TF expression.

Monocytes taken from healthy subjects were preincubated with or without 50 $\mu\text{mol/L}$ vitamin E (α -tocopherol; Sigma-Aldrich, Milan, Italy) for 1 hour and were then stimulated with 0.4 ng/mL LPS (*Escherichia coli* OB11: B4; Sigma, St Louis, MO). TF expression and thrombin generation were measured over 6 and 24 hours of incubation time, respectively, as described below.

Methods

Blood coagulation study. Blood samples anticoagulated with sodium citrate (9:1, vol/vol) were taken from patients who had fasted for at least 12 hours between 8:00 and 9:00 AM. The samples were immediately centrifuged at 2,000g for 20 minutes at 4°C, and the supernatant was collected and stored at -80°C until measurement. 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 nmol/L; range, 0.3 to 1.2 nmol/L).⁴ Intra-assay and interassay coefficients of variation were 8% and 9%, respectively.

Endotoxemia. The test was performed using a chromogenic substrate test (Kabi Diagnostica, Stockholm, Sweden) employing the end-point method, as previously described.⁵ The day-to-day coefficient of variation was 11% (reference value, 4.4 ± 1.5 pg/mL).

Measurement of Isoprostane- $F_{2\alpha}$ -III. Urinary Isoprostane- $F_{2\alpha}$ -III levels were assayed using a stable-isotope dilution gas chromatography/mass spectrometry assay as already described.²⁰ Briefly, a known amount of the internal standard [$^{18}\text{O}_2$] Isoprostane- $F_{2\alpha}$ -III, prepared as previously described by Pickett and Murphy,²¹ was added to each sample. After solid-phase extraction, the samples were purified by thin-layer chromatography and analyzed on a Fison M800 (Fison Instruments, Milan, Italy) gas chromatography/mass spectrometer. Quantification was performed using peak area ratios. Urinary creat-

inines were determined by a standard automated colorimetric assay using a Beckman Synchron CX System (Beckman Instruments, Arlington Heights, IL).

Isolation and incubation of blood mononuclear cells. Peripheral blood mononuclear cells were isolated from the heparinized venous blood of liver cirrhotic patients and controls using aseptic technique. Platelets were removed by using two-step centrifugation, once at 140g and twice at 100g in phosphate-buffered saline (PBS) at room temperature for 10 minutes. Peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation on lymphoprep (Nyegaard, Oslo, Norway) at 1,200g for 20 minutes at 20°C. Monocytes, identified by May-Grunwald-Giemsa staining, comprised 16% to 22% (mean, 19%).

Monocytes (adherent cells) were obtained by incubation of the PBMCs for 90 minutes at 37°C in humidified atmosphere of 5% CO_2 in air in Petri dishes containing RPMI 1640, supplemented with 2 mmol/L glutamine; lymphocytes (nonadherent cells) were removed by aspiration with a Pasteur pipette and washing of the dishes with warm media.²² The purified monocyte preparation contained 85% to 95% monocytes. After isolation, cells were washed twice in PBS and incubated without LPS at 2×10^5 cells/mL in RPMI 1640 at 37°C 5% CO_2 for 6 hours. At the end of the incubation period, the cells and media were separated by centrifugation (2,000g for 15 minutes). The cells were washed with Tris-NaCl buffer (0.1 mmol/L NaCl, 0.1% bovine serum albumin, pH 7.4) and then lysed in the same buffer by adding 15 mmol/L n-octyl- β -D-glycopyranoside at 37°C for 30 minutes.⁹ A cell count and trypan blue exclusion were performed on cell suspensions after washing.

In another set of experiments (see in vitro study above), monocytes (2×10^5 cells/mL) taken from healthy volunteers were preincubated for 1 hour with 50 $\mu\text{mol/L}$ vitamin E or medium as control and then incubated with 0.4 ng/mL LPS in RPMI 1640 at 37°C in 5% CO_2 for 6 hours. At the end of incubation period, the samples were treated as described above.

TF assay. TF activity was determined in the cell lysate by measuring monocyte procoagulant activity with a one-stage clotting assay.²³ Briefly, aliquots (100 μL) of cell lysate were added to 100 μL of normal pooled citrated plasma; after 150 seconds of incubation at 37°C, 100 μL of 0.025 mmol/L CaCl_2 was added and the clotting time was recorded using a Schnitger and Gross coagulometer (Germany). All samples were tested in duplicate. Clotting times were converted to arbitrary TF units per 2×10^5 monocytes using logarithmic plots of clotting times versus dilution of a standard TF solution obtained using commercial thromboplastin (Dade International Inc, Miami, FL). Undiluted thromboplastin was assigned a value of 1,000 TF units, corresponding to a clotting time of 14 seconds. This procoagulant activity was not demonstrated 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, CT). The lower detection limit is approximately 10 pg/mL. The assay recognizes TF-apolipoprotein (TF-apo), TF, and TF-factor VII (TF-VII) complexes and is designed such that there is no interference from other coagulation factors or inhibitors of procoagulant activity.

Thrombin generation rate by LPS-stimulated monocytes. Thrombin generation rate by LPS-stimulated monocytes was evaluated in vitro, by incubating LPS-stimulated monocytes with heparinized standard plasma. For this purpose, a low molecular weight heparin (LMWH) anticoagulated blood sample (ratio, 1:10) was taken from healthy volunteers who gave informed consent to participate in the study. The blood was anticoagulated with LMWH (20 U/mL), because this agent effectively inhibits the formation of thrombin in solution but has only a small effect on thrombin generated at and bound to surfaces.²⁴ Monocytes (2×10^5 cells/mL) taken from healthy subjects were preincubated for 1 hour with vitamin E (50 $\mu\text{mol/L}$) or medium as control and then incubated with

Table 1. Clinical and Laboratory Characteristics of Liver Cirrhotic Patients According to Child-Pugh's Classification

Variable	Grade A (n = 6)	A v B P Value	Grade B (n = 20)	B v C P Value	Grade C (n = 4)	A v C P Value
Age (yr) NS	58 (34-74)	NS	58 (44-79)	NS	63 (56-69)	NS
No. of men (%) NS	4 (67)	NS	13 (65)	NS	2 (50)	NS
Serum albumin (g/dL) H = 10.1, P = .0065	4.15 (3.7-4.7)	.005	2.95 (2.3-4.8)	NS	2.55 (2.2-3.5)	.010
Prothrombin activity (%) H = 11.8, P = .0027	87 (70-97)	.012	69 (37-92)	.020	47 (40-65)	.010
TF antigen (pg/2 × 10 ⁵ monocytes) H = 14.0, P = .0009	7.5 (5-56.4)	.005	41.55 (8-76.8)	.008	89.9 (58-101.8)	.010
TF activity (U/2 × 10 ⁵ monocytes) H = 11.4, P = .0034	2.5 (0-25)	.016	17.5 (0-35)	.015	40.5 (20-50)	.018
Isoprostane-F _{2α} -III (pg/mg creatinine) H = 10.4, P = .0055	108 (92-285)	.024	263.5 (44-432)	.02	600 (260-812)	.019
F1 + 2 (nmol/L) H = 10.2, P = .0062	0.95 (0.6-2.15)	.026	1.82 (0.75-2.4)	.022	3.05 (1.8-3.3)	.019
Endotoxemia (pg/mL) H = 16.9, P = .0002	5.4 (3.1-9.5)	.0006	21.83 (5.8-53.3)	.009	56.7 (23.4-62.0)	.010

Data are expressed as the median, with the range in parentheses.

Abbreviations: H, Kruskal-Wallis test; TF, tissue factor; F1 + 2, human prothrombin fragment F1 + 2; NS, not significant.

LPS (0.4 ng/mL) for 6 hours as described above. The medium was removed and 1,000 μL of overlay heparinized standard plasma was added to each well and incubated at 37°C for 24 hours.²⁵ After the incubation, samples were harvested, centrifuged at 4,000g, and assayed for F1+2 generation, which was calculated from the increase in F1+2 level compared with value obtained in control samples, which consisted of heparinized plasma added with LPS. All samples were assayed in duplicate. The results represent the mean of four experiments.

Statistical Analysis

Statistical analysis was performed using the χ^2 statistic or Fisher's Exact Test for independence. Pairwise analysis was performed as appropriate. Two-tailed tests of significance were used throughout. Correlations were assessed using linear regression analysis. When necessary, log transformation was used to normalize the data, or appropriate nonparametric tests were employed. Data are presented as the median (with the range in parentheses) given the apparent departure of data from distributional normality. The required significance level for all tests was set at .05.²⁶

RESULTS

Urinary excretion of Isoprostane-F_{2α}-III (median [range]: 256 [44-812] v 80 [49-160] pg/mg creatinine; $P < .0001$), F1+2 plasma levels (mean [standard deviation (SD)]: 1.7 [0.7] v 0.6 [0.25] nmol/L; $P < .0001$), monocyte TF antigen (median [range]: 41.5 [5-101.8] v 15.5 [10-39] pg/2 × 10⁵ monocytes; $P < .0001$), and activity (median [range]: 17.5 [0-50] v 9 [4-20] U/2 × 10⁵ monocytes; $P < .03$) were significantly higher in cirrhotic patients than in controls.

The clinical and laboratory characteristics of liver cirrhotic patients divided according to Child-Pugh classification are reported in Table 1. TF antigen ($P = .0009$) and activity ($P = .0034$) and prothrombin fragment F1+2 ($P = .0062$) progressively increased from A to C class (Table 1). A significant correlation was observed between prothrombin fragment F1+2 and TF activity (Rho = 0.89, $P < .0001$) and antigen (Rho = 0.89, $P < .0001$).

A marked increase in Isoprostane-F_{2α}-III was detected in cirrhotic patients, particularly in those with the highest degree of liver failure. Thus, cirrhotic patients of C class had higher values of Isoprostane-F_{2α}-III compared with those of B ($P =$

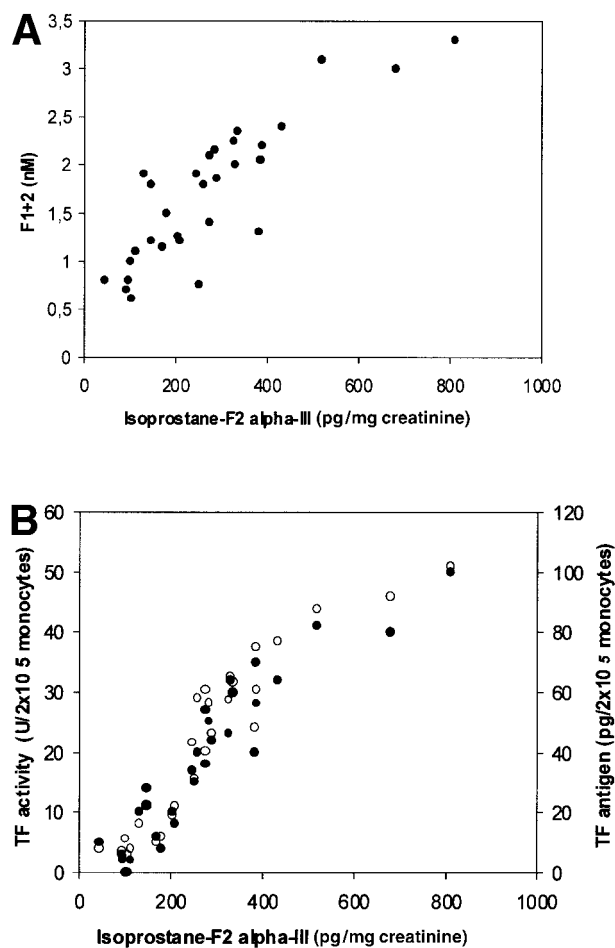


Fig 1. Correlation between prothrombin fragment F1+2 and urinary excretion of Isoprostane-F_{2α}-III in cirrhotic patients (A) and correlation between monocyte tissue factor antigen (○) and activity (●) and urinary excretion of Isoprostane-F_{2α}-III in cirrhotic patients (B).

.02) and A ($P = .019$) classes (Table 1). According to our previous study,¹⁵ we found a significant correlation between Isoprostane- $F_{2\alpha}$ -III and endotoxemia ($Rho = 0.72$, $P < .0001$). Isoprostane- $F_{2\alpha}$ -III was also significantly correlated with F1+2 ($Rho = 0.85$, $P < .0001$; Fig 1A) and TF antigen ($Rho = 0.95$, $P < .0001$) and activity ($Rho = 0.94$, $P < .0001$; Fig 1B), suggesting that lipid peroxidation and clotting system activation are closely related.

To further explore such a relationship, we investigated whether the administration of vitamin E had some effect on lipid peroxidation and clotting activation. At baseline, no difference in clinical and laboratory characteristics was observed between the groups receiving or not receiving vitamin E (Fig 2). After vitamin E administration, cirrhotic patients showed significantly higher values of plasma vitamin E compared with baseline values (median [range]: 13 [11-17] v 17 [14-29] $\mu\text{mol/L}$; $P = .018$). We also found a significant decrease of Isoprostane- $F_{2\alpha}$ -III (median [range]: 355 [170-812] v 240 [142-560] pg/mg creatinine; $P = .008$ [not shown]), monocyte TF antigen (63.6 [10-101.8] v 22.0 [10-66] pg/ 2×10^5 monocytes; $P = .012$) activity (30 [6-50] v 15 [2-30] U/ 2×10^5 monocytes; $P = .008$), and prothrombin fragment F1+2 plasma levels (2.05 [1.15-3.30] v 1.35 [0.90-2.20] nmol/L; $P = .008$; Fig 2A and B). On the contrary, endotoxin serum levels showed similar values before and after vitamin supplementation (me-

dian [range]: 28.7 [3.5-62] v 29.5 [4.5-54] pg/mL; $P = .139$). In patients receiving only standard treatment, no changes in Isoprostane- $F_{2\alpha}$ -III, monocyte TF antigen and activity, and F1+2 levels were observed (Fig 2A and B).

Further support for this finding was provided by in vitro study performed in monocytes taken from healthy subjects. In this experimental model, we tested the effect of 50 $\mu\text{mol/L}$ vitamin E on the LPS-stimulated (0.4 ng/mL) monocyte activation. The results of this experiment are summarized in Fig 3, showing that 50 $\mu\text{mol/L}$ vitamin E significantly reduced the monocyte expression of TF antigen (-52% ; $P = .001$) and activity (-55% ; $P = .003$). This finding likely accounts for the effect of 50 $\mu\text{mol/L}$ vitamin E on the formation of F1+2 mediated by LPS-stimulated monocytes. Thus, this concentration of vitamin E significantly reduced the rate of thrombin generation (-51% ; $P = .025$) in samples containing LPS-stimulated monocytes and heparinized plasma (Fig 4).

DISCUSSION

This study shows for the first time that in cirrhosis lipid peroxidation and clotting activation are related. The urinary excretion of Isoprostane- $F_{2\alpha}$ -III, which is one of the most abundant F_2 -isoprostanes formed under physiological conditions in human,²⁷ was used as marker of lipid peroxidation. Thus, this compound has been reported elevated in several

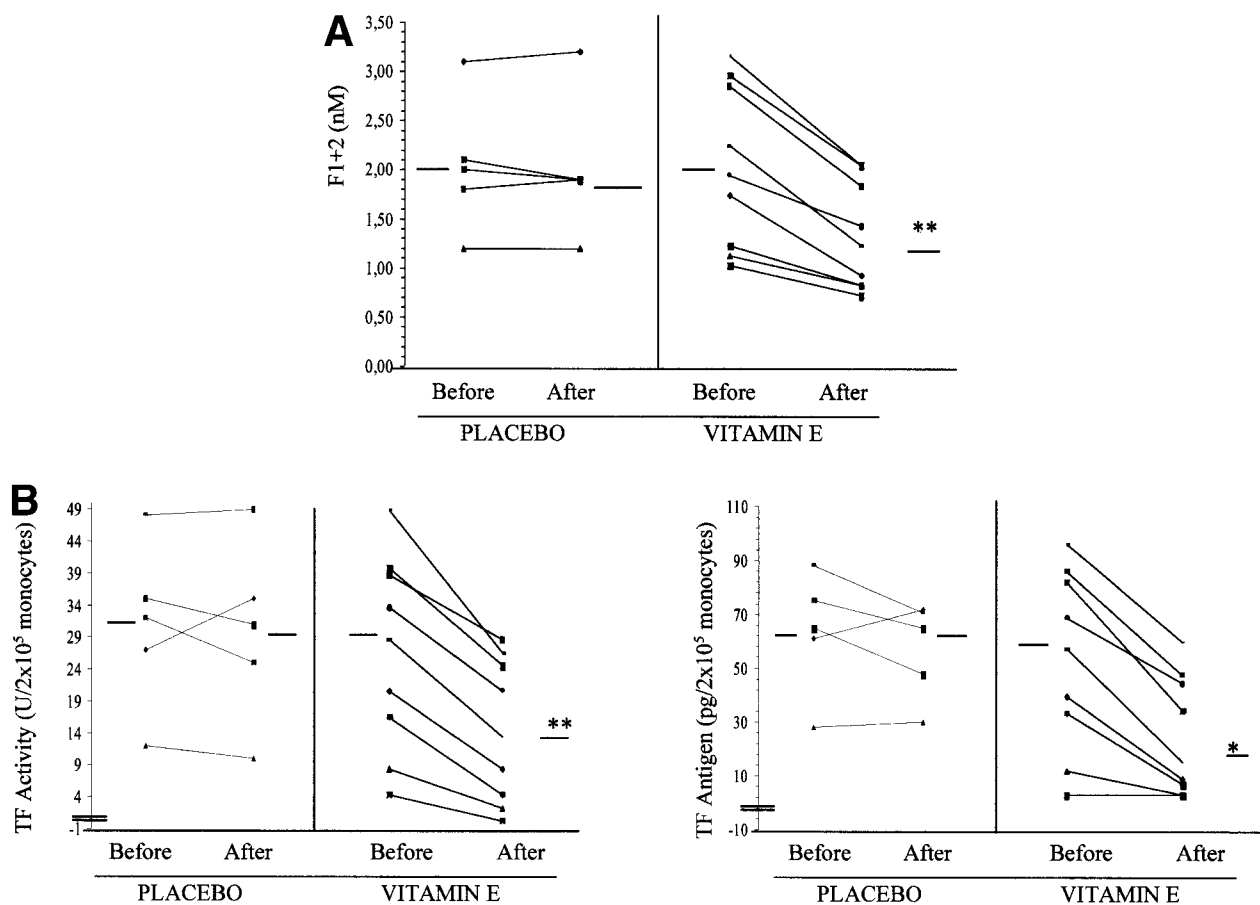


Fig 2. (A) Prothrombin fragment F1+2 plasma levels and (B) monocyte TF antigen and activity in cirrhotic patients before and after vitamin E administration and in placebo group. * $P = .012$; ** $P = .008$. The horizontal lines refer to the median values.

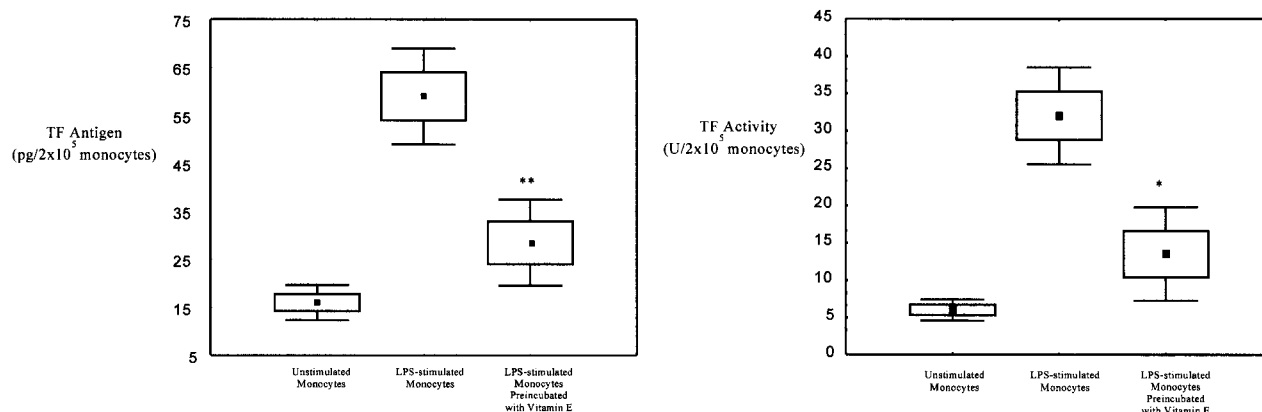


Fig 3. TF antigen and activity expressed by unstimulated or LPS-stimulated (0.4 ng/mL) monocytes added with and without vitamin E (50 μ mol/L). The paired *t*-test was used for statistical analysis. **P* = .003; ***P* = .001. The top and bottom of the box represent the standard error. The square in the middle of the box represents the mean value. The lines extending above and below the box refer to the standard deviation.

pathological conditions associated with oxidant stress such as drug poisoning, cigarette smoking, coronary reperfusion, and autoimmune disease.^{16,17,28,29} Consistent with our previous report,¹⁵ we found elevated urinary excretion of Isoprostane- $F_{2\alpha}$ -III in cirrhosis, particularly in patients with severe liver failure. Such elevation should not be considered artefactual, ie, related to a reduced liver clearance, for several reasons. First of all, experimental and humans' studies demonstrated that liver is poorly involved in Isoprostane- $F_{2\alpha}$ -III clearance.^{30,31} In addition, we demonstrated that vitamin E administration is associated with a significant decrease in Isoprostane- $F_{2\alpha}$ -III, indicating that increase in this isoprostane reflects enhanced formation more than decreased clearance.¹⁵ The relationship between enhanced lipid peroxidation and clotting activation was suggested by several lines of clinical and laboratory evidences. In the cirrhotic population, the urinary excretion of Isoprostane- $F_{2\alpha}$ -III was significantly correlated with plasma levels of prothrombin fragment F1+2 as well as with monocyte TF antigen and

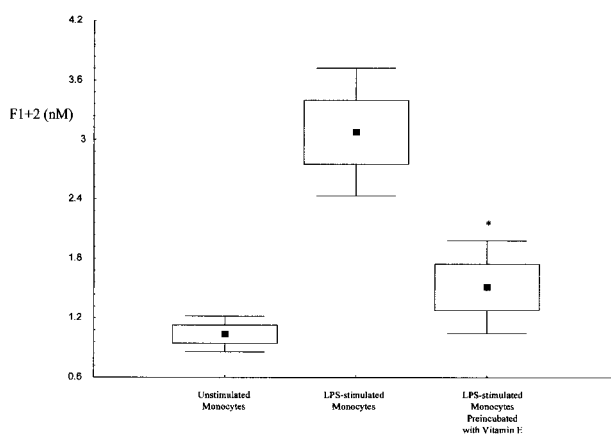


Fig 4. Prothrombin fragment F1+2 generation observed on unstimulated or LPS-stimulated (0.4 ng/mL) monocytes added with and without vitamin E (50 μ mol/L) and exposed to a standard heparinized overlay milieu (see text). The paired *t*-test was used for statistical analysis. **P* = .025. The top and bottom of the box represent the standard error. The square in the middle of the box represents the mean value. The lines extending above and below the box refer to the standard deviation.

activity, suggesting a cause-effect relationship between lipid peroxidation and clotting activation. This hypothesis was explored in an interventional study in which lipid peroxidation and clotting activation were measured before and after vitamin E supplementation. We observed that, after vitamin E administration, F1+2 plasma levels and the monocyte TF expression significantly decreased. It is noteworthy that these changes were not related to endotoxemia, which, in fact, was not modified by the antioxidant treatment. The results of the interventional study were further corroborated by in vitro experiments showing that 50 μ mol/L vitamin E significantly inhibited monocyte TF expression and monocyte-induced F1+2 formation. It is noteworthy that in this experiment monocytes were stimulated with LPS concentration close to that found in the peripheral circulation of cirrhotic patients⁵ and that 50 μ mol/L vitamin E reduced TF expression and F1+2 generation by 50%, on average. This is in accordance with a previous study showing that oxidant species enhance monocyte expression of TF and that 50 μ mol/L vitamin E reduces by approximately 50% LPS-induced monocyte TF expression.³² Our finding also supported the results of an in vivo study showing that, in subjects receiving 1,200 U/d vitamin E, monocyte function is reduced; thus, Devaraj et al³³ demonstrated that, after vitamin E supplementation monocyte formation of oxidant species, lipid oxidation and interleukin-1 β secretion were significantly decreased.

Because of the effect of vitamin E on oxygen free radical formation, it may be postulated that the inhibition of monocyte TF expression is due to its antioxidant property. However, further study is necessary to analyze whether vitamin E has the same effect of other antioxidants, which have been shown to regulate transcriptional or posttranscriptional activation of monocyte TF expression.^{13,14}

Our results may have potential clinical relevance, because the concentration we used in vitro is achievable in human blood after vitamin E supplementation. Therefore, administration of vitamin E could represent an interesting new approach to modulate clotting activation and, in turn, secondary hyperfibrinolysis in this clinical setting. This suggestion has to be confirmed in a larger controlled trial.

In conclusion, this study provides evidence that, in liver

cirrhosis, there is a relationship between lipid peroxidation and clotting activation and suggests that lipid peroxidation may represent an important mechanism that mediates endotoxin-induced monocyte TF expression. The inhibition of clotting system activation by vitamin E may open a new avenue for the treatment of clotting disturbance with antioxidants in this clinical setting.

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