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Endothelial Cell and Hemostatic Activation in Relation to Cytokines in Patients with Sepsis

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Abstract

Sepsis is commonly associated with disturbances of the hemostatic balance. Most of the pathophysiological changes in sepsis are caused by endotoxin acting directly through endothelial injury or indirectly through release of cytokines with procoagulant effects. The relation between cytokines and hemostatic parameters was assessed in 32 patients with sepsis. Prothrombin fragment 1+2 (F1+2), thrombin-antithrombin III complexes (TAT), tissue type plasminogen activator (t-PA) functional and antigen, plasminogen activator inhibitor-1 (PAI-1), plasmin-α_2-antiplasmin complexes (PAP), D-Dimer, thrombomodulin (TM) and von Willebrand factor (vWF) were measured in patients and in 30 healthy subjects. The levels of cytokines TNF-α and interleukin-6 (IL-6) also were determined. A significant increase of F1+2, TAT, PAI-1, PAP, and D-Dimer was observed in septic patients as compared with controls ($p<0.0001$), whereas t-PA activity was significantly reduced ($p<0.01$). The markers of endothelial cell activation TM, vWF, and t-PA antigen also were elevated significantly as compared with the control group ($p<0.01$). Finally, we found a marked increase of TNF-α and IL-6 ($p<0.0001$). Whereas the increase of cytokine levels could be partially responsible for the hemostatic activation, it did not correlate with markers of endothelial activation in patients with sepsis.

Key Words: Sepsis; Hemostasis; Endothelial damage; Cytokines

Sepsis is defined as a systemic inflammatory response to infection associated with the activation of a number of host defense mechanisms including the cytokine network, leukocytes and the hemostatic system [1]. Disseminated intravascular coagulation (DIC), with widespread deposition of fibrin in the microvasculature, is commonly found in septic shock and is linked closely to the development of multiple organ failure [2,3].

Endotoxin and certain cytokines such as tumor necrosis factor (TNF-α), interleukin 1 beta (IL-1β), IL-6, and IL-8 are potent inducers of the above mentioned changes through important modifications in the surface properties of vascular endothelium, which becomes thrombogenic [4–6]. The endothelial activation/damage induced by endotoxin and cytokines initiates the coagulation cascade and alter the balance between activators and inhibitors of the fibrinolytic system, which favors fibrin deposition [7,8]. However, a direct relationship between the cytokine levels and the changes in markers of hemostatic and endothelial cell activation has not been proven. Moreover, different antiendotoxin and anticytokine strategies have not been useful to counterbalance the alterations observed in sepsis (reviewed in ref. [9]).

Abbreviations: TAT, thrombin-antithrombin III complexes; t-PA, tissue type plasminogen activator; PAI-1, plasminogen activator inhibitor; TM, thrombomodulin; vWf, von Willebrand factor; IL-6, interleukin-6.

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The aim of this work was to analyze whether a correlation between the levels of cytokines and markers of endothelial cell and hemostatic activation is present in patients with sepsis.

1. Patients and Methods

1.1. Samples

The study group was composed of 24 men and 8 women with a mean age of 48±15 years, all suffering from sepsis. An age-sex matched group of 30 healthy subjects served as control. At the time of entry into the study patients presented the typical clinical picture with two or more of the following signs: 1) clinical evidence of infection, 2) tachypnea (respiratory rate greater than 20 breaths per minute), 3) tachycardia (heart rate greater than 90 beats per minute), and 4) temperature abnormalities (greater than 38.4°C or less than 35.6°C). Neither DIC (according to standard clinical and analytical criteria) nor septic shock (blood pressure of <90 mm Hg) was present at the time of sampling.

Blood samples were drawn within 24 hours of the onset of sepsis, collected in siliconized vacutainer tubes containing 0.13 M trisodium citrate and put on ice until centrifugation at 3000×*g* for 15 minutes. Aliquots of platelet-poor plasma were stored at −70°C.

Samples for t-PA determination were collected in Stabilyte tubes (Biopool, Sweden) in order to avoid inhibitors interferences.

1.2. Assays

The following hemostatic markers were included: prothrombin fragment 1+2 (F1+2) and thrombin-antithrombin III complexes (TAT) were assayed using commercial ELISA kits (Enzygnost^R F1+2 and Enzygnost^R TAT, Berhingwerke AG, Germany) [10,11]. Tissue type plasminogen activator (t-PA) activity was determined by using a bioimmunoassay (Coatest BIA tPA; Chromogenix, Mölndal, Sweden) [12]. Plasminogen activator inhibitor (PAI-1) activity was measured by an amidolytic assay (Coatest PAI; Chromogenix) [13]. Plasmin-α_2-antiplasmin complexes (PAP) were measured with an ELISA assay previously described in our laboratory by Montes et al. [14]. D-Dimer levels were measured with an ELISA assay (FibrinostiKa FbDP; Organon Teknika, Turnhout, Belgium) [15].

The following markers of endothelial activation were measured using ELISA assays: tissue type plasminogen activator antigen (t-PA Ag) (Tint-Elize t-PA; Biopool, Umeå, Sweden) [16]. Thrombomodulin (Asserachrom thrombomodulin; Diagnostica Stago, Asnières, France) [17]. von Willebrand factor (Asserachrom von Willebrand factor; Diagnostica-Stago) [18].

The levels of cytokines IL-6 and TNF-α were determined using the ELISA Kits Coaliza IL-6 and Coaliza TNF-α (Chromogenix) [19,20].

1.3. Statistical Analysis

The mean values, median and standard error of the mean (SEM) are reported. Values were tested for the type of distribution using Kolmogorov-Smirnov test. Statistical analysis was done with the two-tail Mann-Whitney U test for comparison of median of unpaired data. Correlations were calculated using Spearman's rank test. A value of $p<0.05$ was considered to be significant.

2. Results

Thirty-two patients with sepsis were included in the study, of which 20 presented positive blood cultures. Sepsis was due to Gram negative bacteria in 14 patients (43.7%). Gram positive organisms were detected in five patients (15.6%), and fungal infection was present in 1 patient (3.1%). The remaining 12 patients presented a localized site of infection (*Staphylococcus aureus* and epidermidis and *Escherichia coli*) and negative blood cultures.

Tables 1 and 2 show the mean values of hemostatic and endothelial cell activation markers. Correlations between the different parameters are shown in Table 3.

2.1. Clotting Activation Markers

The markers of activation of the coagulation mechanism, F1+2 and TAT, were significantly elevated in patients with sepsis as compared with the control group ($p<0.0001$), indicating an important degree of prothrombin activation and thrombin genera-

Table 1. Hemostatic parameters in patients with sepsis and controls

	Sepsis	Control	p
F1+2 (nmol/l)	2.08±0.18 (1.89)	1.02±0.11 (1.00)	<0.0001
TAT (ng/ml)	14.05±1.75 (12.00)	1.66±0.05 (1.60)	<0.0001
t-PA activity (U/ml)	0.17±0.03 (0.14)	0.27±0.02 (0.24)	=0.003
PAI-1 activity (U/ml)	23.62±2.04 (23.00)	8.93±0.93 (8.00)	<0.0001
PAP (ng/ml)	1982.49±382.47 (1446.50)	573.50±125.10 (530.00)	<0.0001
D-Dimer (ng/ml)	3641.33±749.15 (2200.00)	240.67±7.78 (260.00)	<0.0001

Mean values ±SEM (median) are indicated.

tion (Table 1). A positive correlation between both markers was observed ($r=0.54$, $p<0.01$) (Table 3).

2.2. Fibrinolytic Components

As regards fibrinolytic parameters we found a significant increase of PAI-1, PAP, and D-Dimer ($p<0.0001$). Conversely, a marked decrease of t-PA activity ($p=0.003$) was observed in the patients group as compared to controls (Table 1). We also observed a negative correlation between t-PA activity and PAI-1 ($r=-0.77$, $p<0.0001$) and positive between PAP and D-Dimer ($r=0.48$, $p<0.05$) (Table 3).

2.3. Markers of Endothelial Cell Activation

Thrombomodulin, t-PA antigen and von Willebrand factor are released after endothelial perturbation. A significant increase of these parameters was observed in patients as compared with controls ($p<0.005$), with maximum differences for t-PA and vWF ($p<0.0001$), indicating an important degree of endothelial activation in septic patients (Table 2).

As shown in Table 3, the following correlations were observed: vWF correlated positively with t-PA Ag ($r=0.50$, $p=0.007$) and TM ($r=0.53$, $p=0.004$). TM also correlated significantly with PAI-1 ($r=0.43$, $p=0.02$) and negatively with t-PA activity ($r=-0.66$, $p=0.001$). Finally, we found correlations of t-PA Ag with PAI-1 activity ($r=0.58$, $p=0.001$) and negatively with t-PA activity ($r=-0.52$, $p=0.009$).

No differences in any of the hemostatic parameters and endothelial cell markers were observed in Gram negative as compared with Gram positive sepsis (data not shown).

2.4. Cytokines

The plasma concentration of TNF-α and IL-6 were assessed in all patients and controls (Table 2). We observed a marked increase of both cytokines in the group of patients ($p<0.0001$), being the elevation of IL-6 five times higher than the increase of TNF-α with respect to the values observed in controls.

We also assessed the correlations between these cytokines and the different parameters analyzed (Table 3). A positive correlation between TAT levels with both TNF-α ($r=0.39$, $p=0.04$) and IL-6 ($r=0.64$, $p<0.01$) could be demonstrated. IL-6 also correlated with D-Dimer ($r=0.42$, $p<0.01$) and PAI-1 activity ($r=0.58$, $p<0.001$). No significant correlations of cytokines with the markers of endothelial activation were observed.

Table 2. Markers of endothelial activation and cytokine levels in patients with sepsis and controls

	Sepsis	Control	p
TM (ng/ml)	102.47±14.88 (56.80)	37.36±3.14 (37.20)	=0.005
vWF (%)	406.27±30.95 (412.50)	101.44±9.59 (99.00)	<0.0001
t-PA Ag (ng/ml)	23.63±3.04 (20.00)	4.76±0.37 (5.00)	<0.0001
TNF-α (pg/ml)	73.61±12.11 (70.00)	5.71±0.69 (5.00)	<0.0001
IL-6 (pg/ml)	186.35±34.33 (103.00)	3.12±0.65 (5.00)	<0.0001

Mean values ±SEM (median) are indicated.

Table 3. Correlations found in patients with sepsis

	F1+2	TAT	t-PA act.	PAI-1 act.	PAP	D-D	TM	vWF	t-PA Ag	TNF-α	IL-6
F1+2		0.54*	−0.35	0.27	0.13	0.55*	0.53*	0.08	0.16	−0.00	0.12
TAT	0.54*		−0.26	0.43**	0.10	0.57*	0.27	0.04	0.32	0.39**	0.64***
t-PA activity	−0.35	−0.26		−0.77***	−0.10	−0.18	−0.66***	−0.71***	−0.52*	−0.33	−0.32
PAI-1 activity	0.27	0.43**	−0.77***		−0.37**	0.13	0.43**	0.32	0.58*	0.32	0.59*
PAP	0.13	0.10	−0.10	−0.10		0.48*	0.09	0.05	−0.20	−0.18	−0.20
D-D	0.55*	0.57*	−0.19	−0.19	0.48**		0.34	−0.00	−0.10	0.27	0.42**
TM	0.53*	0.27	−0.66***	0.43**	0.09	0.34		0.53*	0.23	0.32	0.15
vWF	0.08	0.04	−0.71***	0.3	0.05	−0.00	0.53*		0.50*	0.24	0.02
t-PA Ag	0.16	0.32	−0.52*	0.58*	−0.20	−0.10	0.23	0.50*		0.19	0.23
TNF-α	−0.00	0.39**	−0.33	0.33	−0.18	0.27	0.32	0.24	0.19		0.31
IL-6	0.12	0.64***	−0.32	0.58*	−0.20	0.42**	0.15	0.02	0.23	0.31	

The correlation coefficient (r) was calculated by the Spearman's rank test.
*$p<0.01$; **$p<0.05$; ***$p<0.001$.

3. Discussion

It is known that most of the pathophysiological changes in sepsis are caused by endotoxin acting directly through endothelial injury or indirectly through release of mediators, such as TNF and cytokines, which also affect the endothelial cell function triggering procoagulant and antifibrinolytic effects [4,5,21,22]. The current study was undertaken to determine whether the plasma levels of some markers of hemostatic and endothelial cell activation correlate with the cytokine levels in a series of patients with sepsis.

A significant increase of F1+2 and TAT was observed in patients with respect to controls, indicating a marked clotting activation, similar to that observed after intravenous administration of endotoxin to human subjects [4], probably reflecting activation of the extrinsic pathway as assessed by in vivo studies using monoclonal antibodies to TF and factor VII [23–25].

As regards fibrinolysis, we found a significant increase of PAI-1 activity, PAP, and D-Dimer, as well as a decrease of t-PA activity in patients with respect to controls. The fibrinolytic system seems to play an important pathologic role in sepsis and may contribute to the appearance of microthrombi [26–28]. Several studies have reported that, after endotoxemia and cytokinemia, the fibrinolytic system becomes initially activated and subsequently inhibited [4,22,28]. The injection of TNF-α or endotoxin to humans is initially followed by a strong increase in plasminogen activator activity, which results in the conversion of plasminogen to plasmin, as reflected by the enhanced levels of PAP observed in our series of septic patients. Later, the increased PAI-1 neutralizes t-PA, although the fibrinolytic system still remains functionally active as demonstrated by the generation of D-Dimer. An association of PAI-1 levels with mortality in DIC due to sepsis has also been shown [29].

The lack of differences in the parameters analyzed in relation to blood cultures could be due to the reduced sample analyzed, although it is also known that the hemostatic activation is not always associated with the plasma endotoxin concentrations [4,26].

In a further step, we analyzed several markers of endothelial cell activation, since vascular endothelium plays an important role in the regulation of the hemostatic balance in sepsis [7,30]. The plasma

levels of vWF, TM, and tPA were markedly elevated, indicating a marked endothelial activation [30–32]. The observed correlations between TM with both TAT and t-PA activity would indicate that endothelial perturbation contributes to the clotting and fibrinolysis activation in sepsis.

Laboratory and clinical evidence indicates that the toxic effects of endotoxin are mediated by cytokines, some of which were detected in significant amounts in our series of septic patients, confirming previous reports [20,33,34]. To demonstrate whether the cytokine increase was responsible for some of the observed changes, we analyzed their correlations with markers of endothelial and hemostatic activation. A significant correlation was found between cytokines and hemostatic activation markers, suggesting that inflammatory mediators directly contribute to both thrombin and plasmin generation in human sepsis, which agrees with in vivo studies after administration of endotoxin to healthy subjects [6], as well as experimental studies using specific monoclonal antibodies against TNF and IL-6 [35–37].

The lack of correlations between cytokines and markers of endothelial damage could be due to differences in in vivo half lives (e.g., 5 minutes for t-PA and 12 hours for vWF), as well as to the fact that cytokines exert effects at a distance from the site of production. In addition, the mechanisms responsible for the release of these markers from vascular endothelial cells vary considerably [38,39]. Thus, absence of correlations does not neccesarily mean absence of causality, since local effects cannot be excluded. On the other hand, whether such a correlation would exist in severe sepsis accompanied by shock or DIC has not been explored in the present study.

In conclusion, during sepsis an important hemostatic activation takes place, indicated by a strong increase of F1+2 and TAT. The fibrinolytic system remains functionally active in spite of the enhanced PAI-1 activity. We also found a marked increase of TM, vWF and t-PA Ag, indicating an important degree of endothelial cell activation, and a significant increase of circulating cytokines TNF-α and IL-6. The lack of correlation between cytokine levels and endothelial cell markers does not exclude a pathogenetic role of cytokines in the endothelial cell perturbation present in human sepsis.

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