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IN FOCUS

Platelet function in sepsis

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Summary. *Background*: Coagulation abnormalities and thrombocytopenia are common in severe sepsis, but sepsis-related alterations in platelet function are ill-defined. *Objectives*: The purpose of this study was to elucidate the effect of sepsis on platelet aggregation, adhesiveness, and growth factor release. *Patients and methods*: Agonist-induced platelet aggregation was measured in platelet-rich plasma separated from blood samples collected from 47 critically ill patients with sepsis of recent onset. Expression of platelet adhesion molecules was measured by flow cytometry and the release of vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) was measured by ELISA in the supernatant of platelet aggregation. *Results*: Septic patients had consistently decreased platelet aggregation compared with controls, regardless of the platelet count, thrombin generation, or overt disseminated intravascular coagulation (DIC) status. The severity of sepsis correlated to the platelet aggregation defect. Adhesion molecules, receptor expression (CD42a, CD42b, CD36, CD29, PAR-1), and α-granule secretion detected by P-selectin expression remained unchanged but the release of growth factors was differentially regulated with increased VEGF and unchanged PDGF after agonist activation even in uncomplicated sepsis. *Conclusions*: Sepsis decreases circulating platelets' hemostatic function, maintains adhesion molecule expression and secretion capability, and modulates growth factor production. These results suggest that sepsis alters the hemostatic function of the platelets and increases VEGF release in a thrombin-independent manner.

Keywords: platelet adhesion, platelet aggregation, platelet-derived growth factor, vascular endothelial growth factor.

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Introduction

Platelet function can be seen as a succession of overlapping events involving adhesion, aggregation, secretion, and promotion of procoagulant activity [1,2]. By adhesion to the subendothelium and aggregation, platelets maintain primary hemostasis by forming hemostatic plugs, occluding sites of vascular damage and allowing the assembly of coagulation complexes responsible for thrombin generation [1]. Coagulation abnormalities and thrombocytopenia are common in severe sepsis [3–6], and may range from minor changes in platelet counts and alterations in coagulation tests to full-blown disseminated intravascular coagulation (DIC) and widespread microvascular thrombosis [7]. The severity of hemostatic disorders seems to correlate with the severity of disease; in particular, a low platelet count is predictive of a poor outcome [4,8–10].

Studies on platelet function in sepsis have yielded conflicting results. In a lethal endotoxin shock model in dogs, Almqvist *et al*. [11] reported increased platelet aggregation and others have also shown endotoxin-induced platelet accumulation and enhanced platelet aggregation in animal models [12–14]. *In vitro,* bacterial compounds such as lipopolysaccharide (LPS) and *Staphylococcus aureus* lipoteichoic acid can bind to platelets and endothelial cell membranes of septic patients and inhibit platelet aggregation [15–18]. However, sepsis-generated cytokines do not seem to activate human platelets either directly or via thrombin [17]. In clinical studies, several investigators have reported decreased platelet aggregability during sepsis [18,19], whereas Gawaz *et al*. [20] noted increased platelet aggregability.

Platelet α-granules contain vascular growth factors, such as vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and platelet factor-4 (PF-4), which are released during aggregation [21]. VEGF is a potent angiogenic factor regulating proliferation, differentiation, and survival of microvascular endothelium [22] and PDGF contributes to endothelial repair and angiogenesis [23,24], but little is known about their regulation during the septic response.

The aim of the present study was to analyze the platelets of septic patients including three main platelet functions: aggregation, adhesion and secretion in order better to define sepsis-related alterations in platelet function.

Materials and methods

The study protocol was approved by the Ethics Committee of the Free University of Brussels, Erasme Hospital and the patients or relatives gave informed consent.

Patients and study design

Platelet function was investigated in 47 consecutive septic patients admitted to the medico-surgical intensive care unit (ICU) of a university hospital and in 15 healthy volunteers. Septic patients were defined as having at least one positive blood culture and/or an identified focus of infection. Severe sepsis was defined by at least one organ failure related to sepsis [25] and septic shock was defined as sustained sepsis-related hypotension necessitating the administration of vasopressor agents. Exclusion criteria were: age under 18 years, heparin administration, drugs affecting prostanoid synthesis, and patients with a blood creatinine concentration above 2.5 mg dL^{-1} at entry. Healthy volunteers had no evidence of infection and had not taken a non-steroidal anti-inflammatory drug (NSAID) within the 10 days prior to the study.

The criteria for DIC were those defined by the Subcommittee on Disseminated Intravascular Coagulation of the International Society of Thrombosis and Haemostasis [based on platelet count, soluble fibrin monomers, prothrombin time (PT), and fibrinogen concentration] [26]; a score above 5 was considered as DIC. Severity of disease was assessed according to the Acute Physiology and Chronic Health Evaluation (APACHE) II score [27] and the Sequential Organ Failure Assessment (SOFA) score [28].

Blood samples were obtained on the day of admission to the ICU in patients with sepsis and at the onset of sepsis in patients who developed sepsis during the ICU stay. Twenty milliliters of blood were drawn in sodium citrate (3.8%) tubes (Terumo, Tokyo, Japan) from an arterial catheter after discarding the first 5 mL of blood. Healthy volunteers were sampled by venepuncture.

Thrombin generation assessment

In vivo thrombin generation was assessed by four ELISA assays measuring thrombin–antithrombin (TAT) complex, prothrombin fragment 1 + 2, soluble fibrin monomers (Enzygnost TAT, Enzygnost F1 + 2 and SF; Boehring®, Schwalbach, Germany), and D-dimers (Mini Vidas D DI; Biomerieux®, Marcy l'Etoile, France). Plasma was prepared by 15 min centrifugation at 2000 × *g* at 25 °C and stored at − 80 °C until assayed.

Platelet-rich plasma preparation and platelet aggregation

Whole blood (500 μL) was used for flow cytometry, and platelet-rich plasma (PRP) was immediately prepared from the remaining blood by centrifugation at 90 × *g* for 10 min at 20 °C. Platelet-poor plasmas (PPP) were prepared from PRP by a second centrifugation at 2000 × *g* for 15 min at 20 °C. Part of the PPP was frozen at − 80 °C for later ELISA analysis. The PRP from normal healthy volunteers was sequentially diluted in PPP to match the low platelet counts in PRP of the thrombocytopenic septic patients.

Platelet aggregation was assessed according to the manufacturer's instructions. Briefly, PRP was added to the tube of a four-chamber lumiaggregometer (PAP-4 aggregometer®; Bio/Data Corporation, Horsham, PA, USA) set at 37 °C with constant stirring at 1000 r.p.m. The agonists used were 500 μg mL^{-1} arachidonic acid (Bio/Data Corporation), 5 μM ADP (Roche, Mannheim, Germany), 2.5 μg mL^{-1} collagen (Chrono-Log Corp., Havertown, PA, USA), 70 μM thromboxane A_2 analog (U46619®; Sigma, St Louis, MO, USA) and 50 μM thrombin receptor agonist peptide (TRAP6; Bachem Ag, Bubendorf, Switzerland). Agonist-induced aggregation was recorded graphically for 10 min. Results are given as a ratio of the percentages of aggregation in the septic patients and in the healthy controls with the closest PRP platelet concentrations after dilution.

White blood cells (WBC) hardly contaminated the PRP, and the maximum numbers of WBC and monocytes remaining in the PRP were 150 ± 11 mm^{-3} and 10 ± 1 mm^{-3}, respectively.

Flow cytometry analysis

The samples from the last 10 patients were studied by flow cytometry. In these 10 patients, four had uncomplicated sepsis and six severe sepsis or septic shock; three had DIC. To evaluate platelet aggregability we measured the increased avidity of GPIIbIIIa for fibrinogen with directly fluorescein isothiocyanate (FITC)-stained fibrinogen (Dako, Glostrup, Denmark), and to show conformational change in GPIIbIIIa, we used the specific monoclonal antibody PAC-1 FITC (Becton Dickinson, San Jose, CA, USA). Adhesion molecules were studied using anti-CD42a–FITC (Glycoprotein V), CD42b–phycoerythrin (PE) (Grycoprotein Iα), CD29–FITC (common β-chain of the collagen, fibronectin and laminin receptor) and CD36–FITC (Glycoprotein IV) from Immunotech (Marseille, France). Alpha-granule secretion was studied using CD62p–PE (P-selectin; Becton Dickinson) expression. Protease-activated receptor-1 (WEDE15–PE) (Immunotech) was also tested as the main thrombin receptor in platelets.

Whole blood (5 μL) was added to polypropylene tubes containing mixtures of the monoclonal antibodies. ADP (5 μM), 50 μM TRAP6 and 2.5 μg mL^{-1} collagen were then added and after 15 min incubation at room temperature, the platelets were fixed in 1 mL 1% paraformaldehyde in phosphate-buffered saline (PBS) (Bio Whittaker, Walkersville, MD, USA). Data were acquired using a FACScabilur flow cytometer (Becton Dickinson) and Cell Quest software (Becton Dickinson).

Platelets were defined as events fitting the platelet size and complexity in log scale forward and side scatter and expressing

fluorescence in the FL3 channel using CD61 peridinin chlorophyll protein Per-CP (Becton Dickinson). Stability of the instrument was controlled daily using QC3 beads from Flow Cytometry Standard Corp. (San Juan, PR, USA) and linearity was checked weekly using Rainbow beads (Spherotec, Libertyville, IL, USA) with an interday variation below 2%. The median fluorescence intensity (MFI) was used to depict molecule expression on the platelet surface.

VEGF and PDGF measurement

To evaluate *in vitro* growth factor release, the supernatants of the aggregation induced by TRAP6 and collagen were collected after centrifugation at 2000 × *g* to remove aggregates and free remaining platelets and kept at −80 °C for determination of VEGF and PDGF content by ELISA. Commercially available immunoassay kits (human VEGF immunoassay and human PDGF-AB immunoassay; R&D Systems, Minneapolis, MN, USA) were used. The sensitivity of VEGF was 9 pg mL^{-1} and of PDGF-AB, 8.4 pg mL^{-1}. Optical density at 450 nm was measured on a Microplate Autoreader (Bio-Tek Instrument, Houston, TX, USA) and the concentrations were determined by linear regression from a standard curve. The amounts of VEGF and PDGF released in the PRP per platelet were calculated using the formula: Growth Factors in pg per million platelets = [Growth factor in the aggregated PRP supernatant (pg mL^{-1}) − Growth factor in the PPP (pg mL^{-1})] × 10^6/number of platelets in the PRP sample.

Statistical analysis

All values are expressed as mean ± SD. Data were analyzed by unpaired Student's *t*-test (normal distribution) or a Mann–Whitney *U*-test. Pearson's correlation coefficient was used in correlations. $P < 0.05$ was considered to be statistically significant.

Results

The characteristics of the patients are shown in Table 1. Of the 47 patients, 16 had uncomplicated sepsis, 31 had severe sepsis or septic shock. The most common microorganisms isolated were *Escherichia coli* ($n = 12$), *Staphylococcus aureus* ($n = 11$), *Klebsiella* spp. ($n = 6$), and *Pseudomonas aeruginosa* ($n = 6$). The healthy control group included 11 men and four women with a mean age of 37 ± 8 years and normal platelet counts (180 000–400 000 mm^{-3}).

Platelet aggregation and severity of sepsis

We observed a decrease in platelet aggregation in all the septic patients compared with diluted matched controls, regardless of the agonist used. We divided patients into three groups according to the platelet count: those with normal platelet counts (> 180 000 platelets mm^{-3}; eight sepsis and nine severe sepsis), those with intermediate platelet counts (between 100 000 and 180 000 mm^{-3}; four sepsis and 16 severe sepsis), and those with a low platelet count (< 100 000 mm^{-3}; four sepsis and six severe sepsis). Platelet aggregation was more severely altered in patients with severe sepsis than in those with uncomplicated sepsis, the differences being particularly significant in patients with thrombocytopenia. The most sensitive pathway to sepsis-induced alterations in platelet aggregation was the cyclooxygenase pathway with arachidonic acid (AA) inducing a 50% inhibition of maximal aggregation in patients with a normal platelet count (Fig. 1).

Table 1 Comparison of the characteristics of the septic patients according to the severity of the illness and the occurrence of disseminated intravascular coagulation (DIC) (mean ± SD)

	Severity of illness		Coagulation status	
	Sepsis	Severe sepsis/ septic shock	No DIC	DIC
Number of patients	16	31	27	20
Age (years)	59 ± 11	63 ± 12	63 ± 13	60 ± 16
Male : female ratio	10 : 6	22 : 9	16 : 11	16 : 4
APACHE II score	9 ± 4	15 ± 7*	12 ± 6	15 ± 7
SOFA score	3 ± 2	8 ± 4*	5 ± 3	8 ± 4**
Number of non-survivors	0	25	6	19
Platelet count (× 10^3 mm^{-3})	229 ± 90	145 ± 109	240 ± 96	84 ± 36[a]
Antibiotics	16	39	30	25
Aminopenicillin	6	6	8	4
Cephalosporin	6	10	11	5
Carbapenem	4	14	8	10
Aminoglycoside	0	5	2	3
Vancomycin	0	4	1	3
Vasoactive agents	0	25	12	13
Dopamine	–	13	7	6
Dopamine + norepinephrine	–	5	0	5
Dobutamine	–	6	3	3

*$P < 0.05$ sepsis vs. severe sepsis/septic shock; **$P < 0.05$ no DIC vs. DIC.

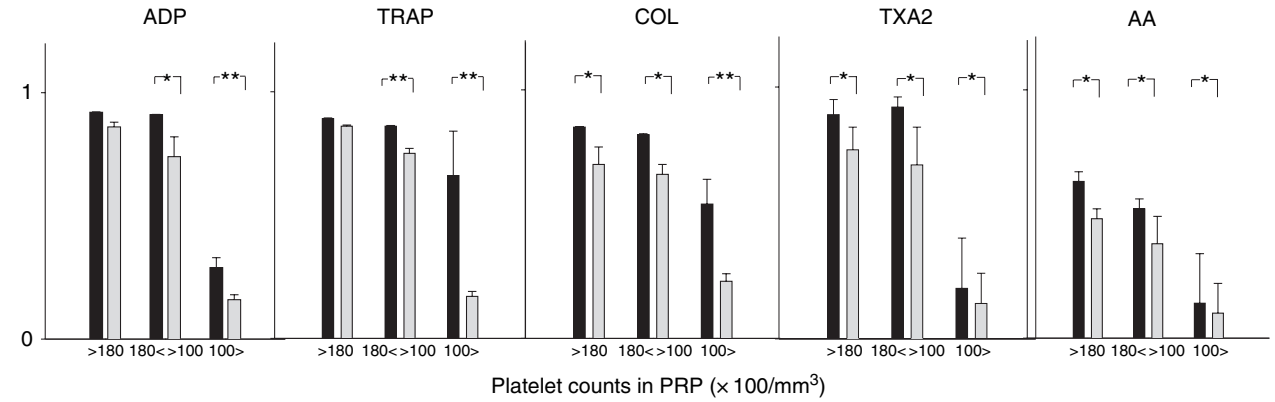

Fig. 1. Maximal platelet aggregation ratio induced by the agonists ADP 5 μM, TRAP 50 μM, collagen 2.5 μg mL^{-1} (COL), thromboxane A_2 70 μM (U46619; TXA2), arachidonic acid 500 μg mL^{-1} (AA) according to the platelet count in the platelet-rich plasma (PRP) (× 100 mm^{-3}) in patients with uncomplicated sepsis (black bars) and severe sepsis/septic shock (gray bars). *$P < 0.05$; **$P < 0.01$.

Table 2 Thrombin generation in 36 patients

	Normal values	All patients ($n = 36$)	Platelet count > 180 000 mm^{-3} ($n = 17$)	180 000 mm^{-3} > platelet count > 100 000 mm^{-3} ($n = 11$)	Platelet count < 100 000 mm^{-3} ($n = 8$)
Soluble fibrin (mg L^{-1})	0–17	27.9 ± 11.3	25.9 ± 7.5	27 ± 15.4	33.5 ± 11.4
D-dimers (ng mL^{-1})	< 500	1882 ± 1152	1521 ± 965	2072 ± 1223	2132 ± 1271
TAT (μg L^{-1})	1.0–4.1	16.0 ± 20.2	10.9 ± 10.6	15.2 ± 14.6	28 ± 35.4
F1 + 2	0–1.1	1.8 ± 0.9	1.8 ± 1.0	2.1 ± 1.0	1.6 ± 0.7

TAT, Thrombin–antithrombin; F1 + 2, prothrombin fragment 1 + 2.

Table 3 Maximal platelet aggregation ratio induced by the agonists in septic patients with and without disseminated intravascular coagulation (DIC)

Platelet count		*N*	ADP	TRAP	Collagen	TXA2	AA
> 180 000 mm^{-3}	No DIC	15	0.81 ± 0.01	0.78 ± 0.01	0.71 ± 0.05	0.89 ± 0.06	0.63 ± 0.06
	DIC	2	0.85 ± 0.03	0.88 ± 0.01	0.8 ± 0.01	0.85 ± 0.09	0.64 ± 0.09
< 180 000 > 100 000 mm^{-3}	No DIC	12	0.84 ± 0.06	0.78 ± 0.01	0.69 ± 0.03	0.86 ± 0.04	0.51 ± 0.08
	DIC	8	0.91 ± 0.002	0.73 ± 0.01	0.74 ± 0.01	0.82 ± 0.15	0.49 ± 0.13
< 100 000 mm^{-3}	No DIC	0	0	0	0	0	0
	DIC	10	0.45 ± 0.48	0.51 ± 0.4	0.43 ± 0.19	0.16 ± 0.14	0.12 ± 0.16

N, Number of patients.

Platelet aggregation and thrombin generation

Thrombin generation was assessed in 36 patients, including 26 with a DIC score between 2 and 5, and 10 patients with a score above 5 (Table 2). There was no correlation between soluble fibrin, D-dimers, TAT and F1 + 2, and the aggregation ratio related to ADP, TRAP and collagen in all patients or just in patients with a normal platelet count. Moreover, platelet aggregation was similarly altered in patients with and without DIC (Table 3).

Flow cytometry analysis

The activated conformation of GPIIbIIIa, assessed by the specific monoclonal antibody PAC-1 or by FITC-stained fibrinogen binding, was decreased in septic patients after platelet activation by TRAP or collagen (Table 4). The study of adhesion molecules by flow cytometry *ex vivo* and after agonist activation revealed no significant alterations in the septic patients. Specifically, the adhesion glycoproteins involved in von Willebrand factor-dependent rolling (CD42a and CD42b), the VLA common β-chain for the fibronectin, laminin, and collagen receptor (CD29), and the thrombospondin receptor (CD36) remained unchanged. PAR-1 expression in the sepsis group was normal, contrasting with the impaired response to TRAP6. Alpha-granule secretion, as evidenced by the expression of P-selection (CD62p) with or without agonist activation, was unaltered in the septic patients.

Release of VEGF and PDGF during platelet aggregation

VEGF released by platelets after agonist activation was significantly increased in all septic patients. Collagen induced a greater VEGF release than TRAP6. There was no

Table 4 Flow cytometry analysis

	Control ($n = 7$)	Sepsis ($n = 10$)
TRAP		
Fibrinogen	744 ± 27	415 ± 152*
PAC-1	481 ± 74	352 ± 85*
CD62p	508 ± 122	424 ± 134
Collagen		
Fibrinogen	566 ± 115	244 ± 153*
PAC-1	334 ± 123	199 ± 124*
CD62p	185 ± 74	127 ± 44

Results are in median of fluorescence intensity on a 1024-channel linear scale (mean ± SD). *$P < 0.05$ vs. control.

difference according to the severity of sepsis or the DIC status. TRAP6-induced VEGF release was even more significant in uncomplicated sepsis than in severe sepsis and in patients without DIC than in those with DIC. In contrast, PDGF release from platelets was not different in sepsis and controls, and independent of the severity of sepsis and the DIC status (Fig. 2). There was no correlation between thrombin generation (assessed by soluble fibrin, D-dimers, TAT and F1 + 2) and VEGF or PDGF release following platelet activation by TRAP6 or collagen regardless of the patient's platelet count.

Discussion

Platelet function involves platelet adhesion, aggregation, secretion, promotion of procoagulant activity, and induction of vascular healing by growth factor release. In the present study we show that platelet functions can be regulated separately in sepsis, with alterations in aggregation that contrast to the maintenance of adhesion molecule expression, the conserved α-granule secretion, and the modulation of growth factor contents with increased VEGF release.

We excluded patients suspected of having primary or acquired platelet dysfunction or underlying disease such as myelodysplasia or myeloproliferative disorders, and those receiving NSAIDs, heparin, or steroids, to avoid any potential interference with the results. However, we could not avoid antibiotic treatment and antibiotics may play a role in platelet aggregation dysfunction. For example, penicillins are known to increase bleeding time mainly by interfering with ADP-induced platelet aggregation [29].

In agreement with some previous reports [18,19], we observed decreased agonist-induced platelet aggregation in the septic patients. Moreover, flow cytometry confirmed this aggregation defect, demonstrating concomitant alteration in the GPIIbIIIa integrin conformational changes with decreased avidity for the PAC-1 antibody and fibrinogen binding in the platelets of the septic patients. These changes were observed even in patients with a normal platelet count or a low DIC score and were completely independent of thrombin generation as measured by soluble fibrin monomers, TAT and F1 + 2, or coagulation as shown by the D-dimer levels. Although platelet aggregation can require fibrinogen [30], platelet aggregation was independent of the amount of thrombin generated or the DIC status in our patients, but rather related to the severity of the sepsis. We do not believe that platelets were exhausted because they could express other conserved functions such as α-granule secretion or VEGF production. Regardless of the severity of sepsis, platelets also preserved normal expression of all the adhesion molecules, suggesting that the ability of platelets to adhere is probably preserved. This is consistent with other studies [20]. Finally, the preserved expression of the

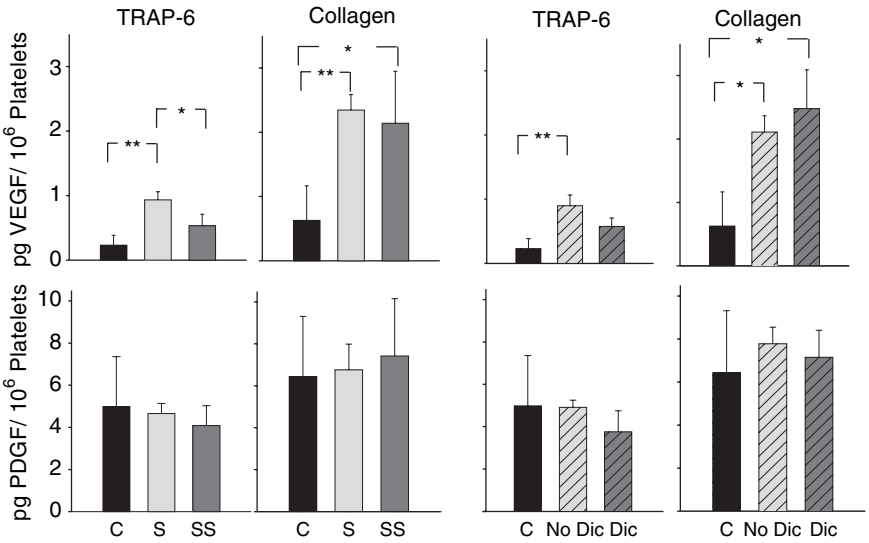

Fig. 2. Concentrations of vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) (in pg/10^6 platelets) in the supernatant of aggregation after administration of TRAP 50 μM or collagen 2.5 μg mL^{-1}. Left panels; subgroups uncomplicated sepsis (S); $n = 16$ (light gray bars), severe sepsis/septic shock (SS); $n = 31$ (dark gray bars). Right panels; subgroups without disseminated intravascular coagulation (DIC) (No DIC); $n = 27$ (light gray hatched bars) and with DIC (DIC); $n = 20$ (dark gray hatched bars). Both panels; healthy controls (C); $n = 15$ (black bars). *$P < 0.05$; **$P < 0.01$.

high-affinity collagen receptor evidenced by the expression of CD29, and the thrombin receptors (PAR-1), even when aggregation induced by these agonists was profoundly affected, evokes a defect at the intracellular transduction pathway level rather than a downregulation of surface receptors. Our study indicates that the cyclooxygenase pathway may be particularly altered in this sepsis-related dysfunction. In accordance with these findings, Lundahl *et al*. [8] observed that platelets had decreased fibrinogen binding capability in response to arachidonic acid, which was correlated with a poor outcome.

In contrast to aggregation, α-granule secretion function was not modified by the severity of the sepsis and we found no *in vivo* increased P-selectin (CD62p) expression on the circulating platelets and the agonist-induced expression of CD62p was preserved on all the septic patients' platelets tested. Similar results have been reported by Gawaz *et al*. [20]. However, Salat *et al*. observed higher expression of CD62p in septic patients, but with a low level of statistical significance [31]. As VEGF and PDGF are stored in the α-granules, we would expect the release of these angiogenic factors to be maintained. Unexpectedly, we observed a complex regulation with an increased release of VEGF compared with control associated with an unchanged release of PDGF. This imbalance already occurred in the platelets of patients with uncomplicated sepsis, no DIC, low thrombin generation, and normal platelet count.

Altogether, these data suggest that sepsis preserves platelet secretory function but that α-granule content is altered. These changes seem to be related more to the severity of sepsis than to coagulation or thrombin generation. Since platelets have no nucleus, this observation suggests that changes in α-granule content may occur at the megakaryocyte level, probably as a result of the inflammatory response [32]. Thus, before thrombin-related platelet consumption and exhaustion, platelets show decreased aggregability, preserved expression of adhesion molecules, and increased VEGF release, suggesting that sepsis, even when uncomplicated, induces a redistribution of platelet function from hemostasis toward other functions, including vascular healing.

In conclusion, we found that sepsis induces a complex regulation of platelet function, which occurs even when the platelet count is normal, and which is independent of other coagulation abnormalities.

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