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Tissue Factor in Patients With Acute Coronary Syndromes
Expression in Platelets, Leukocytes, and Platelet-Leukocyte Aggregates

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Objective—Activated platelets and circulating platelet-leukocyte aggregates (PLA) are significantly higher in patients with unstable angina than in those with stable angina (SA). Platelets from healthy subjects express TF on activation. The aim of this study was to investigate the expression of TF in PLA, in platelets, and in monocytes of acute coronary syndrome (ACS) patients compared to SA patients and healthy subjects (Controls).

Methods and Results—We enrolled 26 consecutive patients with ACS, 29 patients with SA, and 25 Controls. A significantly greater number of total and TF positive platelet-monocyte aggregates was found by flow cytometry in blood of ACS patients than in either SA patients (3-fold) or Controls (5-fold). ACS patients also had a significantly higher amount of TF-positive platelets than SA or Controls (>3-fold) and significantly higher thrombin generation capacity. TF mRNA expression in platelets was significantly higher in ACS patients than in SA or Controls.

Conclusions—In ACS patients the greater expression of TF in platelets and PLA strengthens the link between platelet activation, blood coagulation, and thrombus formation and may further contribute to the hypercoagulability associated with the disease. (***Arterioscler Thromb Vasc Biol* 2008;28:000-000.)**

Key Words: platelets ■ platelet-leukocyte aggregates ■ tissue factor ■ acute coronary syndrome ■ thrombosis

The acute coronary syndrome (ACS) is a clinical state induced by the thrombosis consequent on the rupture of an unstable atherosclerotic plaque. In this syndrome the procoagulant content of complex plaques triggers both platelet activation and coagulation pathways.[1]

Activated platelets and circulating platelet-leukocyte aggregates (PLA), a sensitive marker of in vivo platelet activation,[2] plaque instability, ongoing vascular thrombosis, and inflammation,[3] have been found to be significantly higher in patients with unstable angina than in those with stable angina (SA).[4,5] It is widely recognized that the interaction between platelets and leukocytes leads to phenotypic changes in both cell types and to the secretion of a variety of bioactive compounds.[6] The contribution, however, of platelet-leukocyte interactions to atheromatous plaque instability and to acute progression of ACS is still under investigation.

Tissue Factor (TF) is the main cellular initiator of blood coagulation, and it is also currently considered the protein that links proinflammatory and prothrombotic mechanisms in the progression of atherosclerosis.[7] Expression of TF by leukocytes[8] or by PLA[9] may trigger the extrinsic coagulation cascade; the thrombin thus generated can activate platelets and leads to the formation of a platelet-fibrin thrombus. Our group and others have recently shown that platelets not only express TF protein,[10–12] but they also contain its specific mRNA which has been shown to be translated into protein.[13,14] In addition, platelet reactivity to classical agonists results in the expression of functional TF on the platelet surface.[10]

Previous studies have shown that plaque-associated TF, TF plasma levels, as well as TF expressing monocytes are higher in ACS than in SA.[15,16] However, no information is available on the expression of TF in platelets or in PLA in ACS.

To gain further insight into the inflammatory/prothrombotic phenotype of ACS patients, we studied the expression of TF in PLA, in platelets, and in monocytes of ACS patients compared to SA patients and healthy subjects.

Materials and Methods

Patient Population

Twenty-six consecutive patients with non-ST elevation ACS (mean age 65±10 years; 21 men), and 29 patients with SA (mean age 64±10 years; 21 men) were included in the study. Twenty-five clinically healthy subjects (mean age 52±8 years; 16 men) recruited from the health-care staff served as Controls (see Table 1). All

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Table 1. Characteristics of the Study Groups

	ACS (n=26)	SA (n=29)	Controls (n=25)
Age, yr	65±10‡	64±10‡	52±8
Male, n (%)	21 (81)‡	21 (72)†	16 (64)
Platelet count $\times 10^3/\mu l$	237±67	231±85	222±67
WBC count $\times 10^3/\mu l$	7.03±2.19†	6.91±1.91†	5.85±1.14
Risk factors, n (%)			
Hypertension	11 (42)	16 (55)	0
Smoking	5 (19)	8 (28)	4 (16)
Diabetes	4 (15)	4 (14)	0
No. of coronary vessels with stenosis>75% (%)			
0	3 (12)	4 (14)	...
1	11 (42)	10 (34)	...
2	5 (19)	4 (14)	...
3	7 (27)	11 (38)	...
Previous MI, n (%)	4 (15)	8 (28)	0
Previous PTCA, n (%)	5 (21)§	0	0
Previous CABPG, n (%)	5 (21)	2 (7)	0
TnI +, n (%)*	18 (69)	...	...
TnI +, ng/ml	0.38±0.15	...	...
CRP, mg/l	7.7±9‡#	3.6±4.3‡	1.2±0.8
TF, pg/ml	110±10.6‡#	84.2±6‡	69.7±5.6
Medication, n (%)**			0
β-blockers	6 (23)§	17 (59)	
Nitrates	11 (42)	13 (45)	
Calcium antagonists	6 (23)	13 (45)	
Aspirin	9 (35)§	23 (79)	
ACE inhibitors	4 (15)	4 (14)	
Statins	2 (8)	6 (21)	

Data are presented as numbers (percentages) and mean±SD. WBC indicates white blood cells; PTCA, percutaneous transluminal coronary angioplasty; MI, myocardial infarction; CABPG, coronary artery bypass graft surgery; TnI, troponin I; CRP,C-reactive protein; TF, Tissue Factor.

*at hospital admission.

**for ACS patients medication refers at hospital admission.

†$P \leq 0.05$ vs Controls; ‡$P \leq 0.01$ vs Controls; #$P \leq 0.05$ vs SA; §$P \leq 0.01$ vs SA.

participants gave written informed consent and the study protocol was approved by the Ethical Committee of the Institution. For details please see supplemental materials, available online at http://atvb.ahajournals.org.

Blood Sampling

Please see supplemental materials.

Flow Cytometry Analysis

Whole blood (WB) analysis of PLA as well as of PLA-, platelet-, and monocyte-associated TF was performed by 3-color flow cytometry with a fluorescence-activated-cell sorter (FACS) Calibur (Becton Dickinson) equipped with a 15-mW, air-cooled, 488-nm argon-ion laser. The sensitivity of fluorescence detectors was set and monitored using CaliBRITE beads (Becton Dickinson) according to the manufacturer's instructions.

For PLA and monocyte analysis the procedure was as follows: 100 μL of WB was incubated for 15 minutes at room temperature (RT) in the dark with saturating concentration of the following mouse anti human monoclonal antibodies (MoAbs): CD45-PerCp (Becton Dickinson cat no. 345809) to identify leukocyte populations according to cell granularity and fluorescence, CD41-PE (Instrumentation Laboratories, cat no. A07781) to identify platelet adherent to leukocyte, and TF-fluorescein isothiocyanate (FITC) (American Diagnostica, cat no. 4507CJ). FITC-, PE-, and PerCp-conjugated isotype controls were used in all the experiments to quantify the background labeling. Samples were then resuspended in 1 mL of FACS Lysing Solution (Becton Dickinson) and immediately subjected to flow cytometry. A total of 5000 granulocytes and 1000 monocytes per sample were analyzed. PLA were identified as the percentage of positive events (% of Upper Right, %UR) for both leukocyte and platelet-specific markers ($CD45^+/CD41^+$). The FITC-positive events within PLA account for the percentage of TF-positive aggregates. The FITC-positive events within monocytes (identified by their forward- or side-scatter properties/$CD45^+/CD41^-$) account for the percentage of TF-positive monocytes.

Platelet analysis was performed as previously described.[10] Briefly, 5 μL of WB was added to PBS (final volume 100 μL) with saturating concentration of mouse antihuman TF-FITC and mouse antihuman CD41-PE MoAbs for 15 minutes at RT in the dark. The samples were then diluted in 600 μL of PBS and immediately analyzed by FACSCalibur. TF positive platelets were determined in 10 000 CD41 positive events per sample. Mean Fluorescence Intensities (MFI, arbitrary units) were calculated from fluorescence histograms for the gated population.

All the data were analyzed with CellQuest Software (Becton Dickinson). The intra- and interassay coefficients of variation were <8% and <15% for MFI and %UR determination respectively.

Thiazole Orange Labeling of Reticulated Platelets

Please see supplemental materials.

Preparation of Washed Platelets

For platelet-rich plasma (PRP) preparation, blood was centrifuged at 160*g* for 15 minutes at RT. The PRP was subjected to 2 consecutive centrifugations to remove contaminating leukocytes. To this aim, the top third of the PRP was collected, placed in a fresh tube, added with 4 μmol/L prostaglandin (PG) E_1 and 5 mmol/L EDTA, and centrifuged at 160*g* for 15 minutes at RT. Then, the top third was collected again and washed twice in HEPES buffer pH 7.45 (10 mmol/L HEPES, 134 mmol/L NaCl, 2.9 mmol/L KCl, 1 mmol/L $MgCl_2$, 12 mmol/L $NaHCO_3$, 0.4 mmol/L Na_2HPO_4, 0.1% glucose, and 0.1% BSA). Pellets were resuspended in TRIS buffer (TRIS-HCl 50 mmol/L, pH 7.9, NaCl 100 mmol/L, EDTA 10 mmol/L, BSA 1 mg/mL), assessed for leukocyte contamination, and only the preparations that meet requirements described below were used for thrombin generation measurement or for RNA extraction.[10]

Assessment of Leukocyte Contamination

Please see supplemental materials.

Preparation of Mononuclear Cells

Please see supplemental materials.

Preparation of Megakaryocytes

Please see supplemental materials.

Measurement of TF, P-Selectin, and CRP

Please see supplemental materials.

Generation of Thrombin

Please see supplemental materials.

RNA Extraction and Real-Time Polymerase Chain Reaction

Please see supplemental materials.

Statistical Analysis

Data are expressed as mean±SEM unless otherwise indicated. Groups were compared using ANOVA with Tukey's post test. Adjustment for age, gender, and drug treatment was performed by covariance analysis (ANCOVA) on log-transformed data. Only

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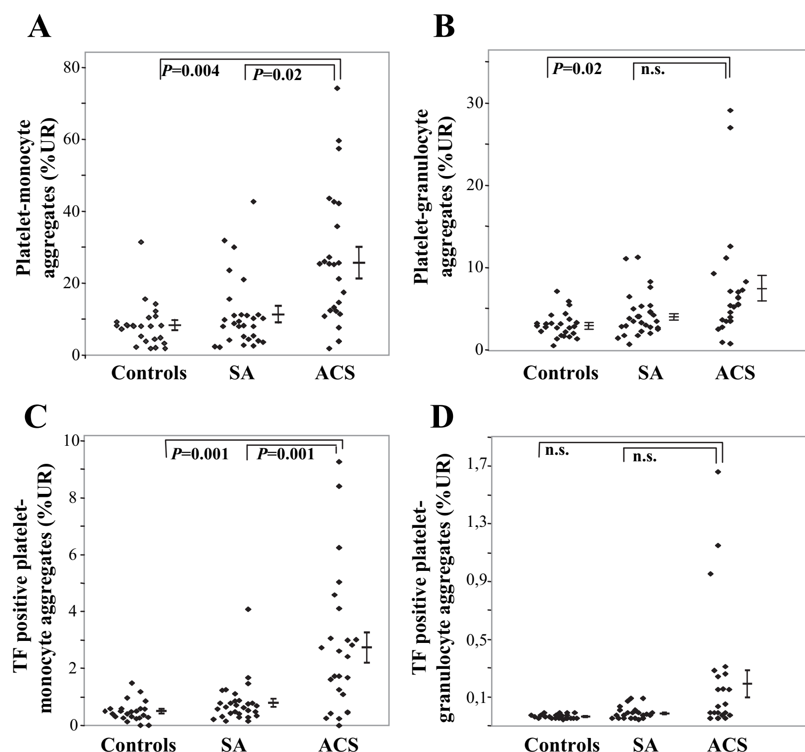

Figure 1. Total and TF-positive PLA are elevated in peripheral blood of ACS patients. WB flow cytometry analysis of total platelet-monocyte (A), platelet-granulocyte aggregates (B), and TF positive platelet-monocyte (C) and platelet-granulocyte aggregates (D) in Controls, SA, and ACS patients. Data are expressed as % of positive events (%UR). See Methods for details.

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$P<0.05$ was regarded as statistically significant. All analyses were performed using the SAS statistical package v8.02 (SAS Institute).

Results

Table 1 summarizes the demographic, clinical, and angiographic characteristics of the patients enrolled in the study. The 2 groups of patients were similar with respect to age, gender, risk factor profile, and platelet and WBC counts. As expected, CRP and TF plasma levels were significantly higher in ACS patients. All patients were using various combinations of drugs such as beta blockers, nitrates, etc. None of the Controls was taking any therapeutic drugs.

Markers of Platelet Activation Are Elevated in ACS Patients

Plasma levels of P-selectin and the percentage of PLA circulating in peripheral blood were measured as markers of platelet activation. As expected, plasma P-selectin was twice as high in ACS (72±27.3 ng/mL, $P=0.003$ versus Controls) and 1.5 times in SA patients (58.5±18.5 ng/mL, $P=0.02$ versus Controls) as in Controls (36.9±2.9 ng/mL). By flow cytometry, a significantly greater number of platelet-monocyte aggregates was found in peripheral blood from ACS patients than from either SA or Controls (2 and 3 times, respectively) (Figure 1A). Platelet-granulocyte aggregates were also significantly higher in ACS patients than in Controls (Figure 1B).

TF Expression in PLA, Monocytes and Platelets Is Elevated in ACS Patients

The percentage of TF-positive platelet-monocyte aggregates was significantly higher in ACS than in SA patients (more than 3-fold higher) and in Controls (5-fold higher) (Figure 1C). TF-positive platelet-granulocyte aggregates showed a trend similar to that observed for TF-positive platelet-monocyte aggregates, being higher in ACS patients than in the other groups; the scanty number of events occurring in each subject, however, does not allow a proper statistical evaluation (Figure 1D).

Because, as previously mentioned, both activated platelets and monocytes can express TF on their surface, it would have been of interest to determine how much TF is expressed by each type of cell in the aggregates. Flow cytometry, however, does not allow to assign TF measured within the aggregate to either cell type. Thus, we further characterize the pattern of TF expression in whole blood measuring TF associated with pure monocyte ($CD45^+/CD41^-$) and platelet ($CD41^+/CD45^-$) populations.

Blood from ACS patients contains a significantly higher number of monocytes expressing TF than that from SA patients and Controls (%UR: 4.85±0.55; 2.46±0.46; 1.76±0.62 respectively, $P=0.009$ ACS versus Controls and $P=0.04$ ACS versus SA). Further, a significant greater amount of TF-positive platelets was also found in ACS patients than in SA patients or Controls (more than 3-fold in both cases) (Figure 2A). Not only was the percentage of TF expressing platelets higher in ACS, but the TF-specific MFI was also significantly higher in ACS than in SA patients and Controls (roughly twice in both cases), indicating that the number of TF molecules expressed on each platelet is significantly higher in ACS than in SA patients and Controls.

The absolute number of TF positive cells and aggregates present in 1 μL blood volume in ACS, SA, and Controls is reported in Table 2.

At multivariate analysis, drug treatment with aspirin and calcium antagonists was associated with lower number of circulating PLA, TF-positive PLA, and TF-positive monocytes.

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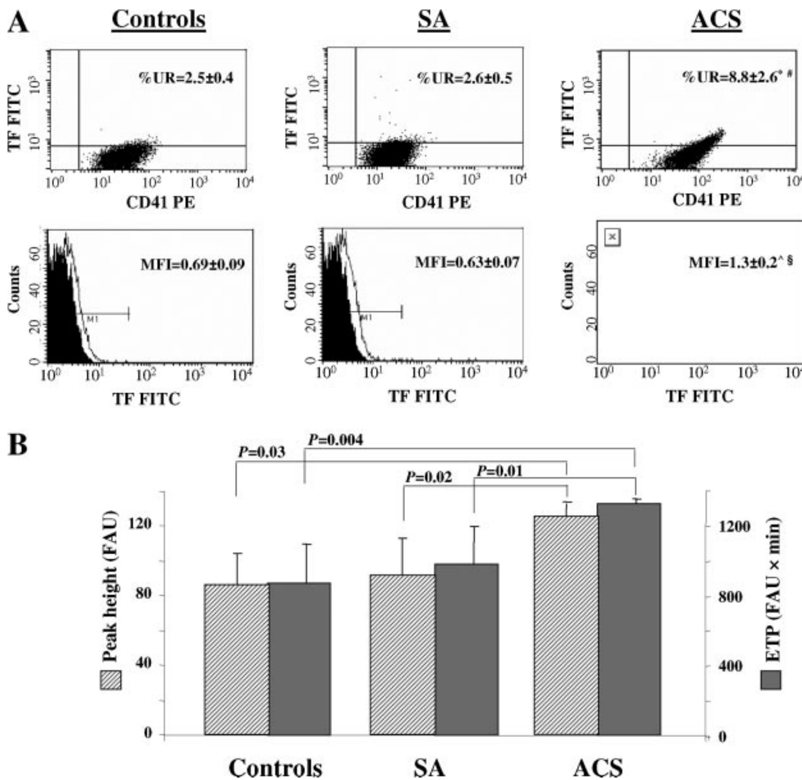

Figure 2. Platelet-associated TF expression and thrombin generation capacity is higher in ACS patients. A, WB flow cytometry analysis of TF expression in platelets from ACS (n=26), SA (n=29) patients, and Controls (n=25). Data are expressed as %UR (*P=0.005 ACS vs Controls; #P=0.004 ACS versus SA) as well as MFI ($\hat{P}$=0.04 ACS vs Controls; §P=0.01 ACS vs SA) as indicated. B, The functional activity of platelet-associated TF in ACS and SA patients and Controls (n=10 for each group) was determined by measuring the thrombin generation capacity with the Calibrated Automated Thrombography in washed platelet lysates (1.0×10^8/mL). The endogenous thrombin potential (ETP; black bars) and the peak height (dashed bars) were both significantly higher in ACS than SA patients and Controls. FAU=Fluorescence Arbitrary Units.

The Platelet-Associated Thrombin Generation Capacity Is Elevated in ACS Patients

The functional activity of platelet-associated TF was determined by measuring the thrombin generation capacity in platelet lysates. The endogenous thrombin potential (ETP) and the peak height were both significantly higher in ACS patients than SA (P=0.01 and 0.02, respectively) and Controls (P=0.004 and 0.03, respectively) (Figure 2B).

Experiments performed in the presence of a specific anti-TF antibody or an anti—tissue factor pathology inhibitor (TFPI) antibody confirmed the partial contribution of platelet-associated TF to thrombin generation (40% reduction and 30% increase respectively; online Figure I).

Increased TF mRNA Expression in Platelets and Monocytes From ACS Patients

We have previously shown that platelets from healthy subjects contain TF mRNA which can even be translated into protein.[10,13,14] Platelets are anucleated cells and derive their RNA content from megakaryocytes. Indeed, we report here that human megakaryocytes isolated from cord blood contain consistent amount of TF mRNA (22±1.8 relative expression compared to TF mRNA expressed in platelets from healthy donors).

Measurement of TF mRNA expression in leukocyte-free platelets (supplemental Figure II) by real-time PCR showed significantly higher expression of the transcript in platelets from ACS patients (3.11±0.51 relative expression, P=0.03 versus Controls) compared to SA and to Controls (1.88±0.78 and 0.7±0.1 relative expression, respectively; Figure 3A).

Of interest, TF mRNA levels in ACS significantly correlated with CRP levels (r=0.61, P=0.035).

Table 2. TF-Positive Cells and Aggregates in ACS, SA, and Controls in 1-Microliter Blood Volume

	ACS	SA	Controls
Platelets	20952±6190	6047±1163	5556±889
Monocytes	14±1.5	7±1.3	4±1.4
Platelet-monocyte aggregates	8±1.7	2±0.4	1±0.02

Values represent the mean±SD of TF-positive cells determined by using the flow cytometry data and the platelet and WBC counts.

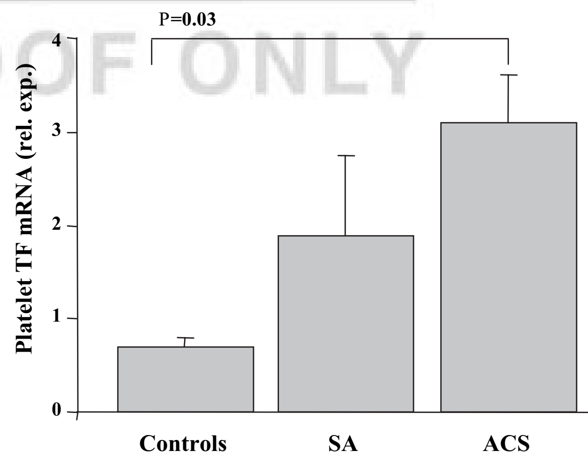

Figure 3. Expression of TF mRNA is higher in platelets from ACS patients. TF mRNA levels in platelets of ACS (n=11), SA (n=13) patients, and Controls (n=10) were determined by real-time PCR. Amplification of GAPDH and β-actin mRNA was used to normalize for differences in RNA extractions and amplifications. Histograms represent the TF mRNA relative expression (±SEM) calculated on a standard curve. Samples were run in triplicate and amplifications were confirmed three times.

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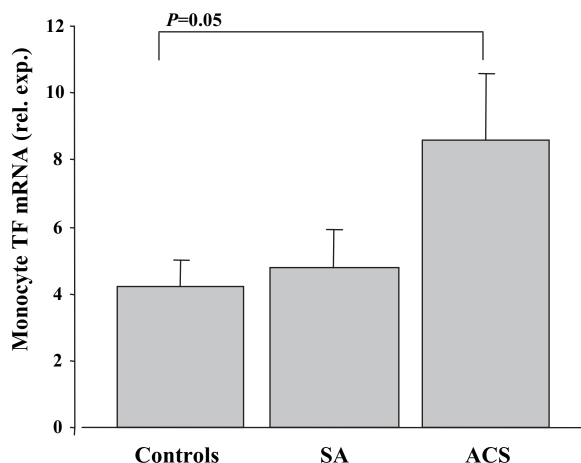

Figure 4. Expression of TF mRNA in monocytes is higher in ACS patients. TF mRNA levels in monocytes of ACS (n=11), SA (n=13) patients, and Controls (n=10) were determined by real-time PCR. Amplification of GAPDH and β-actin mRNA was used to normalize for differences in RNA extractions and amplifications. Histograms represent the TF mRNA relative expression (±SEM) calculated on a standard curve. Samples were run in triplicate and amplifications were confirmed 3 times. See Methods for details.

It is known that platelet turnover may be higher in ACS patients, and newly synthesized platelets contain more mRNA than old platelets.[17] Mean platelet volume, an index of newly released platelets, did not differ significantly among ACS, SA, and Controls (7.3±0.8 fL in ACS, 6.6±0.7 in SA, 6.8±0.4 in Controls), and fully concordant data were obtained with the evaluation of reticulated platelets (RP) by thiazole orange staining (% RP: Controls: 6.9±0.7; SA: 7.6±0.6; ACS: 7.3±0.5).

TF mRNA expression in monocytes showed a pattern similar to that observed in platelets within the groups being significantly higher in ACS patients compared to Controls (Figure 4).

Discussion

In this study we show for the first time that the numbers of TF-positive platelets and TF-positive platelet-monocyte aggregates circulating in ACS patients are significantly higher than those in SA patients and in healthy subjects, providing a further explanation of the increased cell thrombogenicity in ACS.

Patients with coronary artery disease (CAD) have long been reported to have circulating activated platelets, platelet-derived microparticles, platelet-monocyte aggregates, and increased platelet reactivity.[5,18] Platelets are nowadays recognized as playing a major role in inflammation as well as in hemostasis and thrombosis, being the source of inflammatory mediators and able to both produce and respond to chemoattractant cytokines.[19,20] The binding of platelets or platelet-derived microparticles to monocytes (it is not possible to discriminate between platelet and platelet microparticles bound to leukocytes by flow cytometry) in ACS is one of the clues to the interaction of inflammation and thrombosis: when inflammation begets local thrombosis, this in turn exacerbates inflammation, resulting in a vicious circle.[1,21]

We now provide evidence that in ACS patients have more than 3 times as many circulating platelets expressing TF on their surface as are found in SA patients and in Controls. Not only is the number of TF-positive platelets higher in ACS, but each platelet expresses twice the number of TF molecules than in the other groups studied, so that the total capacity to generate thrombin is greater. Thus, TF-bearing platelets as well as PLA might be responsible not only for the increase in thrombus formation triggered by unstable plaque rupture, but also may provide potential distal sites for the generation of new thrombi.

Although further studies are needed to determine in vivo the relative contribution of platelet-associated TF to thrombotic events, we believe that this finding is of particular importance especially if we consider the absolute number of circulating platelets and that of circulating monocytes in an adult subject. In 1 μL blood volume there are roughly 200×10^3 platelets and 240 monocytes (considering monocytes 4% of total leukocytes). The percentage of TF positive platelets and monocytes in ACS patients accounts for 8.8 and 4.8%, respectively, and this results in almost a 2000-fold higher amount of TF positive platelets circulating in blood compared to TF positive monocytes (TF positive platelets: $\approx 21 \times 10^3$; TF positive monocytes: 14 in 1 μL blood volume; see also Table 2).

We believe that such a big difference has to be carefully considered. We have not performed in the present study a direct comparison of TF activity between platelets and monocytes, and this may be a limitation of the study. Certainly, the TF activity of a single monocyte is higher than that of a single platelet, but if we consider the number of platelets present in a thrombus we might not underestimate their contribution. Further studies, perhaps in animal models, may help solving this issue.

Another major finding of the present study is the observation that the levels of the specific TF mRNA in platelet devoid of leukocyte contamination from ACS patients are higher compared to that found in SA patients and in Controls. Quantitative differences in selected mRNA levels between acute and stable CAD patients have been also recently observed in platelet transcriptome studies.[22]

Data published in the past few years have shown that platelets possess megakaryocyte-derived mRNAs that can be translated into proteins because young platelets also contain rough endoplasmic reticulum and polyribosomes.[23] Activated platelets synthesize inflammatory proteins such as Bcl-3[24] and interleukin (IL)-1,[25] enzymes,[26] receptors,[27,28] and recently the synthesis of TF has also been reported.[13,14] We have previously shown that platelets from healthy individuals contain TF mRNA,[10] and in the present study we further provide evidence for the presence of TF mRNA in human megakaryocytes.

Several mechanisms may be responsible for the increased TF mRNA levels observed in ACS patients. The presence, as previously described,[13,14] of TF pre-mRNA which could be spliced more efficiently to mature message in platelets from ACS patients could account for this finding. Further studies however are needed to test this hypothesis because in our

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experimental conditions we did not detect TF pre-mRNA in platelet nor in megakaryocytes.

The positive correlation found between TF mRNA and CRP levels may suggest that systemic inflammatory reaction present in ACS patients might affect the TF content in megakaryocytes and consequently in platelets. Finally, an increased stability of TF mRNA through mechanisms involving RNA-binding proteins such as HuR could also be assumed. Appropriate studies however are required to test these hypothesis. Irrespective of the mechanisms involved, the higher amount of TF mRNA found in platelets from ACS patients further strengthen their prothrombotic potential, especially in view of the platelet biosynthetic potential. Of note, we did not find correlation between platelet-associated TF protein expression and TF mRNA levels. This may suggest that other mechanisms, such as a TF-positive microparticle transfer, may also contribute to the TF protein content in platelets.[29]

The platelet-derived TF may contribute to fibrin formation and to the propagation and stabilization of a thrombus but can also participate, as recently shown, in several cellular processes that stimulate atherogenesis such as angiogenesis and cell migration, both of which are associated with plaque growth and, under certain circumstances, plaque weakening leading to destabilization of the lesion.[30] All together these observations are of particular relevance also in the light of the finding that activated platelets and platelet-leukocyte aggregates exacerbate atherosclerosis.[31]

Antiplatelet therapy is reasonably effective in the treatment of ACS, but present forms of antiplatelet therapy fail to prevent coronary events in a substantial proportion of patients.[32,33] This suggests that the complex mechanisms involved in platelet activation and thrombus formation are not completely understood. While aspirin and calcium antagonists treatments were found to be associated with reduced levels of circulating PLA, TF-positive PLA, and TF-positive monocytes (in agreement with previous studies[34,35]), the association with TF expression in platelets was not observed. We have previously shown that aspirin has no effect on agonist-induced membrane exposure of TF,[10] whereas P_2Y_{12} receptor antagonists completely prevent its expression[36] (Marina Camera, unpublished data, 2005) and GPIIbIIIa antagonists, paradoxically, increase the stimulatory effect of adenosine 5′-diphosphate.[10] None of the patients in this study was receiving ticlopidine/clopidogrel or any GPIIbIIIa antagonists. As this was an observational study, the associations we have observed should not be interpreted as cause and effect. Randomized studies are in progress to assess the effect of antiplatelet drugs on platelet TF expression in ACS patients.

In conclusion, the present study further extends the proinflammatory/prothrombotic phenotype of ACS patients showing that new players on the scene, ie, TF-positive platelets and TF-positive PLA, may be seen to contribute to the higher procoagulant potential that is characteristic of these patients.

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Disclosures

None.

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