Platelet Activation Induces Cell-Surface Immunoreactive Tissue Factor Expression, Which Is Modulated Differently by Antiplatelet Drugs

Marina Camera, Marta Frigerio, Vincenzo Toschi, Marta Brambilla, Francesca Rossi, David C. Cottell, Paola Maderna, Alessandro Parolari, Roberto Bonzi, Ombretta De Vincenti, Elena Tremoli

Objective—Tissue factor (TF) is the main activator of the coagulation cascade occurring in physiologic and pathologic conditions. Recent data suggest that human platelets might contain TF that is possibly derived from leukocytes. In this study, we investigated whether intraplatelet TF can be exposed on the membrane by platelet agonists. The modulation of this process by antiplatelet drugs has been evaluated as well.

Methods and Results—Flow cytometric analysis of unstimulated platelets showed a small amount of membrane-associated immunoreactive TF (irTF) in whole blood, platelet-rich plasma, and washed platelets isolated from healthy subjects. ADP, thrombin receptor–activating peptide, and epinephrine significantly increased functionally active, membrane-associated irTF. ADP induced irTF exposure in a concentration- and time-dependent fashion. Agonist-induced irTF expression was completely inhibited by iloprost but not by aspirin. Interestingly, glycoprotein IIB/IIa antagonists did not inhibit but rather potentiated the stimulatory effect of ADP on platelet irTF expression. Real-time polymerase chain reaction experiments showed detectable amounts of TF mRNA in unstimulated platelets.

Conclusions—These findings indicate that platelet agonists and antiplatelet drugs might modulate platelet-associated irTF expression. Regulated TF expression establishes the potential for a previously unrecognized role for platelets in sustaining thrombus formation and growth via coagulation-mediated mechanisms. (Arterioscler Thromb Vasc Biol. 2003;23:1690-1696.)

Key Words: platelets ■ tissue factor ■ coagulation ■ thrombosis ■ antiplatelet agents

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Clinical and experimental evidence indicates that tissue factor (TF), a 47-kDa glycoprotein, triggers activation of the coagulation cascade that occurs in several human diseases, such as sepsis and other systemic conditions. In addition, TF has been shown to be present in atherosclerotic plaques. In particular, TF protein and activity have been found to be increased in coronary plaques, thus playing a crucial role in human coronary syndromes. Vessel wall–associated TF, however, does not entirely explain the thrombogenic potential of vascular lesions when they are exposed to flowing blood. It has been proposed that thrombus growth might be promoted by circulating (ie, microparticle or platelet-associated) TF. Indeed, to interact with plasma coagulation proteins and thus sustain thrombus growth, TF might diffuse from its source to the initial platelet layer. Because the time to capture any diffusing species increases as the square of distance, the shortened distance resulting from TF associated with circulating platelets might have very significant effects on subsequent reaction velocities. Considerable data now support the hypothesis that platelets actively modulate the propagation of coagulation by expressing specific, high-affinity receptors for coagulation proteases, zymogens, and cofactors that contribute to localize thrombin generation at the site of vascular injury. It has been recently reported that platelets, under well-controlled experimental conditions, contain functionally active TF, which might derive from leukocytes through a particle transfer mechanism.

In the present study, we report for the first time that activation of human platelets by the agonists ADP, thrombin receptor–activating peptide (TRAP), and epinephrine induces TF expression on the cell membrane in a time- and concentration-dependent manner, thereby making TF capable of interacting with plasma factor VIIa (FVIIa) and generating FXa. Moreover, we show that this expression is modulated by some, but not all, antiplatelet agents. We also provide evidence that platelets, although they are anucleated blood cells, contain TF mRNA, thus making them capable of

Received April 8, 2003; revision accepted June 4, 2003.

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Arterioscler Thromb Vasc Biol is available at http://www.atvbaha.org DOI: 10.1161/01.ATV.0000085629.23209.AA
potentially synthesizing this protein. Thus, regulated TF expression establishes the potential for a previously unrecognized role for platelets in sustaining thrombus formation via coagulation-mediated mechanisms.

Methods

Materials

ADP and epinephrine were from Sigma, TRAP was from Bachem AG, and human recombinant FVIIa (hrFVIIa) was kindly provided by Novo Nordisk. Human FX and an FXa chromogenic substrate (Spectrozyme) were from American Diagnostica Inc. Aspirin was from Bayer, iloprost was from Italfarmaco, epibatidine was from Schering-Plough, abeciximab was from Ely Lilly, and tirofiban was from Merck Sharp & Dohme. All other reagents were of high commercial grade.

Antibodies

Fluorescein isothiocyanate (FITC)–labeled monoclonal antibody (MoAb) anti-human TF was from American Diagnostica (MoAb No. 1). Two additional anti-TF MoAbs, 1 from Calbiochem (MoAb No. 2) and the other kindly provided by Prof Y. Nemerson (Mount Sinai School of Medicine, New York, NY; MoAb No. 3) were also used. FITC-conjugated anti-human P-selectin MoAb and phycoerythrin (PE)-conjugated anti-human glycophorin (GP) Ib/IIIa MoAb (CD41) were from Instrumentation Laboratories. Peridinin chlorophyll protein (PerCP)-conjugated anti-human CD45 and FITC-, PE-, and PerCP-labeled IgG1 isotypic antibodies were from Becton Dickinson.

Blood Collection and Purification of Platelets

Blood was collected by venipuncture of the antecubital vein of healthy volunteers who did not take antiplatelet drugs within 10 days before blood donation and who gave their informed consent to participate in the study. After the first 4 mL was discarded, whole blood (WB) was drawn with a 19-gauge needle without venous stasis into citrate-containing (1/10 volume of 0.129 mol/L sodium citrate) evacuated tubes (Vacutainer, Becton Dickinson). For platelet-rich plasma (PRP) preparation, WB was centrifuged at 160g for 15 minutes. To avoid leukocyte contamination, the top third of the PRP was aspirated, placed in fresh tubes, and centrifuged as previously described. The top third of the PRP was aspirated and used as the source of platelets for the experiments. Platelet counts were determined with a hemocytometer (Coulter Ac T Diff, Beckman Coulter). Leukocyte contamination was <1 leukocyte/10⁷ platelets as assessed by cell counting and flow cytometric analysis of platelet preparations with CD45 MoAb. Blood was also collected from 2 type I Glanzmann thrombasthenic patients who had been classified as such on the basis of functional tests and platelet membrane GPIIb/IIIa measurement by flow cytometry.

Washed platelets (WPs) were obtained by albumin density-gradient separation, as previously described. For RNA preparation, PRP obtained from 100 mL of blood and mixed with an equal volume of 15.4 mmol/L Tris-HCl, 2 mmol/L EDTA, 5 mmol/L glucose, followed by the addition of 1 μmol/L prostaglandin E₁, was centrifuged at 200g for 6 minutes and washed twice with the same buffer. Pellets were then dissolved in guanidinium thiocyanate buffer and extracted with phenol/chloroform.

Leukocyte Preparation

Leukocyte separation and total RNA extraction were performed according to standard procedures, as previously described. In brief, erythrocytes and buffy coats obtained after the first centrifugation during PRP preparation were diluted 2 times in RPMI 1640 containing 10% sodium citrate and carefully layered on top of a commercially available solution (Ficoll-Paque, Pharmacia Biotech AB). After centrifugation for 20 minutes at 530g, the interphase with leukocytes was collected; washed twice in phosphate-buffered saline (PBS) containing 5 mmol/L EDTA and 5 mmol/L glucose; centrifuged at 400g for 10 minutes; lysed in guanidinium thiocyanate solution; and stored at −80°C until RNA extraction.

Platelet Preparation for Flow Cytometry

WB, PRP, and WPs were diluted with HEPES-Tyrode’s buffer (137 mmol/L NaCl, 2.8 mmol/L KCl, 1 mmol/L MgCl₂, 12 mmol/L NaHCO₃, 0.4 mmol/L Na₂HPO₄, 0.35% bovine serum albumin (BSA), 10 mmol/L HEPES, and 5.5 mmol/L glucose), pH 7.4, to obtain 2×10⁷ platelets per microliter. Samples were stimulated for 15 minutes, if not otherwise indicated, at room temperature with ADP, TRAP, or epinephrine without stirring. Then, platelets were incubated for 15 minutes with saturating concentrations of the FITC–, PE–, or PerCP-conjugated antibodies and analyzed by flow cytometry. When the effect of drugs was tested, platelets were pretreated for 15 minutes and then stimulated with ADP. In selected experiments, platelets were pretreated for 1 hour with pyruvate (200 μmol/L) or vehicle and then stimulated with ADP.

Analysis of Platelets by Flow Cytometry

Flow cytometric analysis of platelets was performed with a flow cytometer (FACSCalibur, Becton Dickinson) equipped with a 15-mW, air-cooled, 488-nm argon-ion laser. In WB sample analysis, leukocytes were excluded with a combination of size- and platelet-specific marker (CD41) gating. The FITC-positive events (TF–, P-selectin) were determined in 10,000 CD41-positive platelets per sample. Mean FITC and PE fluorescence intensities were calculated from fluorescence histograms for the gated population, and data were analyzed by computer (CELLQuest software, Becton Dickinson). The results are expressed as mean fluorescence intensity (MFI).

Immunoelectron Microscopy: Preembedding Method

PRP was incubated with MoAb No. 1 (10 μg/mL) for 15 minutes at 37°C with or without ADP (10 μmol/L). Then samples were fixed with 2% paraformaldehyde in PBS for 15 minutes at room temperature, centrifuged at 800g for 15 minutes, and washed twice with PBS containing 50 mmol/L glycine. Suspensions were immunogold labeled by incubation for 1 hour with a secondary goat anti-mouse IgG conjugated to10-nm gold particles (1/40 dilution in PBS, 0.1% BSA, and 0.1% Tween 20; BB International). Negative controls were established by using only the secondary gold-labeled antibody. Samples were then centrifuged, washed in PBS/BSA/Tween, fixed in 1% glutaraldehyde-PBS, postfixed in 1% OsO₄, dehydrated, and embedded in Epon by using standard methods. Ultrathin sections were stained with uranyl acetate followed by lead citrate. Electron photomicrographs were acquired with a transmission electron microscope (JEOL 200EX), calibrated for accurate magnification, with an acceleration potential of 80 kV and objective aperture of 20 μm. Six hundred cells per sample were examined at various magnifications.

FITC-FVIIa Labeling

For FVIIa flow cytometry binding studies, labeling of hrFVIIa with FITC (30 μg FITC per milligram protein) was carried out overnight at 4°C in conjugation buffer (0.5 mol/L carbonate/bicarbonate, pH 9.5). Unconjugated FITC was then removed by Sephadex G-25 chromatography.

TF Activity Assay

The functional activity of platelet-associated TF was determined by measuring FXa generation in WPs, both in unstimulated as well as in ADP-stimulated cells (10 μmol/L, 15 minutes). At the end of incubation, 20 μL of WP suspension was incubated in duplicate in 96-well plates with FVIIa (2 mmol/L) and CaCl₂ (25 mmol/L) for 15 minutes at room temperature. Human FX (200 nmol/L) was then added, and the samples were incubated for 30 minutes at 37°C. The reaction was stopped by addition of 20 μL EDTA buffer (50 mmol/L Tris-HCl, 20 mmol/L EDTA, 1 mg/mL BSA). Then 10 μL of a chromogenic substrate (Spectrozyme FXa) was added and absorbance at 405 nm was measured. The amount of FXa generated from the platelet suspension was compared with that obtained from a serial
dilution of relipidated hrTF (molecular weight, 30,000) under the same conditions. To prove the TF dependence of FXa generation, experiments were performed in the absence of FVIIa as well as after preincubation (30 minutes at room temperature) of the samples with a specific anti-TF neutralizing antibody (American Diagnostica No. 4509).

**Enzyme-Linked Immunosorbent Assay**

TF antigen was determined by ELISA (Imubind Tissue Factor, American Diagnostica) in unstimulated WPs lysed with PBS containing 1% Triton X-100, 1 mmol/L phenylmethylsulfonyl fluoride, 100 U/mL aprotinin, and 5 mmol/L benzamidine. As a positive control of TF expression, human umbilical vein endothelial cells (HUVECs) were used after stimulation for 6 hours with tumor necrosis factor-α (TNF-α; 10 ng/mL) and processed as described earlier.

**SDS-PAGE and Western Blot Analysis**

Lysates of WPs were prepared, separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis under reducing conditions, transferred to nitrocellulose membranes, and analyzed by Western blotting with MoAb No. 2 (1 μg/mL) as previously described.12 As a positive control of TF expression, HUVECs were used after stimulation for 6 hours with TNF-α (10 ng/mL) and processed as described earlier.

**RT-PCR**

Total RNA (100 ng) was reverse transcribed by using 20 U reverse transcriptase (RT; Stratascript, Stratagene) and 0.5 μg oligo(dT) primer together with the manufacturer’s buffer, dNTP (500 nmol/L), and 40 U RNase inhibitor. Incubation was carried out at 42°C for 60 minutes. First-strand cDNA (4 μL) was subjected to 38 cycles of polymerase chain reaction (PCR; denaturation at 94°C for 30 seconds; annealing at 58°C for 30 seconds; and primer extension at 72°C for 60 seconds) in a 100-μL reaction mixture containing 5 U DNA polymerase (YieldAce, Stratagene) and 200 nmol/L sense and antisense primers, which were designed according to human TF, GPIb, CD2, and CD20 sequences (Table I; see the online data supplement at http://atvb.ahajournals.org). PCR was performed on a thermal cycler (Perkin-Elmer GeneAmp PCR system 2400). To show that signals obtained from PCRs were reverse transcriptase dependent and not caused by contamination with genomic DNA or cDNA, RNA was reverse transcribed in the presence or absence of retrotranscriptase. PCR products were analyzed on 2% agarose gels containing 0.1 μg/mL ethidium bromide.

**Quantitative Real-Time PCR**

Real-time quantitative PCR was performed on commercially available system (iCycler Optical System, Bio-Rad Laboratories). Primers for TF and 18S ribosomal RNA were designed with the use of Primer Express (PE Applied Biosystems). Amplicons of 100 to 200 bp with

**Figure 1.** Detection of TF. A, 120 μg of WP lysates was diluted in sodium dodecyl sulfate sample buffer and analyzed by Western blotting for the presence of TF. HUVECs (40 μg) stimulated with TNF-α were loaded as a positive control. Result is representative of 3 experiments. B, ADP induces irTF and (C) P-selectin expression on platelet surfaces. WB, PRP, and WPs were incubated for 15 minutes with buffer (black bars) or 10 μmol/L ADP (dotted bars) and then analyzed by flow cytometry. *P<0.0001, #P<0.01 vs control. D, PRP was incubated for 15 minutes with buffer (black bar) or 0 μmol/L ADP (dotted bars) and then analyzed by flow cytometry with 3 different MoAbs (see Methods). E, FITC-labeled hrFVIIa binds to irTF exposed on platelet surfaces. PRP treated for 15 minutes with buffer (black bars) or 10 μmol/L ADP (dotted bars) was incubated with MoAb No. 1 or with FITC-labeled hrFVIIa and then analyzed by flow cytometry. FITC labeling of hrFVIIa decreased its affinity to TF, therefore explaining the lower binding observed when compared with that obtained with anti TF antibody. **P<0.01, *P<0.05 vs control.
a melting temperature between 68°C and 85°C were selected. Total RNA (0.1 to 1 μg) was reverse transcribed, and PCRs were performed according to the manufacturer’s instructions (Invitrogen Life Technology). Linear amplification of serial 10-fold dilutions of TF plasmid (highest concentration, 2.24 pg/μL) was used as a standard to determine the amount of TF mRNA. Amplification of 18S ribosomal RNA was used to correct for fluctuations in input RNA levels and efficiencies of reactions.

**Statistical Analysis**

Data were analyzed by ANOVA. For evaluation of the effects of drugs, 3 independent factors, eg, type of sample (WB or PRP), presence or absence of ADP, and presence or absence of the antiplatelet drugs used, were considered. Pairwise differences between single groups were tested by Student’s t test with Bonferroni correction for multiple testing. Values of irTF expression were computed as a percentage of unstimulated platelet values.

**Results**

**Platelets Contain irTF**

irTF was detected in resting platelets by Western blot analysis as a specific 47-kD band (Figure 1A). Quantification of the protein by ELISA indicated that lysates of WPs contained 40 times less TF compared with TNF-α-stimulated HUVECs (0.05±0.008 and 2.00±0.1 pg/μg cellular protein, respectively).

**Platelet Agonists Induce irTF Expression on the Cell Membrane**

**Flow Cytometry Analysis**

Unstimulated platelets examined by flow cytometry in WB, PRP, and in isolated WP populations after extensive washing exhibited a small but detectable amount of irTF on their surfaces. After ADP stimulation (10 μmol/L), a significant increase in irTF was observed on platelet membranes (WB: 3.4±1.2 vs 5.55±1.37 MFI, n=20; PRP: 2.8±0.6 vs 4.8±1.3 MFI, n=33; and WPs: 4.2±1.1 vs 6.9±1.9, n=6; all P<0.01; Figure 1B). The finding that platelet-associated TF was comparable in the 3 platelet preparations rules out its possible artifactual increase due to sample manipulation. The increase in irTF paralleled that of P-selectin (Figure 1C). The absence of leukocyte contamination in PRP and WPs was confirmed by the lack of staining of platelet preparations for panspecific leukocyte CD45 antigen, as analyzed in the platelet and the leukocyte size ranges, a result that supports data obtained by blood cell counting (not shown).

To rule out the possibility of nonspecific binding of the anti-TF antibody used, 2 additional anti-TF antibodies (see Methods) were tested. The binding of the 3 different antibodies to membrane-associated TF did not statistically differ (172±14, 138.6±23, and 190±20 MFI for MoAb Nos. 1, 2, and 3, respectively; n=4; Figure 1D). The exposure of TF protein on platelet surface was further confirmed by experiments showing the binding of this protein to its physiologic ligand FVIIa, both under basal conditions as well as on ADP stimulation (Figure 1E). This also suggests that platelet-associated TF might be functionally active.

ADP induced a concentration- and time-dependent increase in irTF expression on platelet membranes (Figure 2A and 2B). Specifically, irTF was detected on platelet surfaces as early as 3 minutes after ADP stimulation, and a constant increase was then observed up to 30 to 60 minutes. TRAP and epinephrine also significantly increased irTF on platelet membranes. Interestingly, irTF surface expression induced by epinephrine was not accompanied by a consistent increase in P-selectin expression. Moreover, the concomitant addition of
ADP and epinephrine to WB or PRP more markedly increased irTF compared with either agonist alone (Figure I; see the online data supplement available at http://atvb.ahajournals.org).

**Electron Microscopy Studies**

To confirm the presence of TF on the surface of platelets, transmission-mode immunoelectron microscopy was performed on both ADP-stimulated and unstimulated platelets. TF, represented by the presence of 10-nm gold particles, was localized to the cell membrane and was observed only in ADP-stimulated platelets (Figure 3). Further studies are needed to better characterize the intracellular distribution of TF; it is worth mentioning, however, that flow cytometry analysis of permeabilized resting platelets showed a significant increase in antibody binding compared with nonpermeabilized platelets (6.3±1.2 vs 2.2±0.4 MFI, respectively; n=3, P<0.01). This suggests the existence of an intracellular pool of TF that agonists stimulation renders available on the platelet surface.

**Platelet-Associated TF Is Functionally Active**

To assess whether irTF exposed on platelet surface triggers the blood coagulation cascade, hrFVIIa-FITC binding was evaluated in PRP. ADP (10 μmol/L) significantly increased FVIIa binding to platelet surfaces (Figure 1D), thus suggesting that platelet-associated TF might be functionally active and therefore, potentially able to generate FXa. Indeed, FXa generation experiments showed that ADP-stimulated platelets were able to form 2.25±0.9 nmol/L FXa during the incubation period. To further prove the contribution of TF in FX cleavage, FXa generation was also performed in the presence of a TF-neutralizing antibody. In these experimental settings, FXa generation was 0.95±0.2 nmol/L, a value that is comparable to that obtained in unstimulated platelets (0.9±0.2 nmol/L FXa). It is worth mentioning that also in the absence of FVIIa, FXa generation was 1.1±0.2 nmol/L. TF content of the samples, calculated as described in Methods, was 0.1±0.02 and 0.26±0.1 pmol/L in unstimulated and ADP-stimulated platelets, respectively (P<0.05).

**TF mRNA Is Present in Human Platelets**

De novo protein synthesis has been shown to occur in human platelets, indicating that metabolically stable mRNAs derived from nucleated megakaryocytes are present in circulating platelets.13,14 RT-PCR experiments showed that resting platelets contained TF mRNA (Figure 4A, lane 2). The coamplification of GPIbα mRNA clearly proved the platelet specificity of the TF mRNA (Figure 4A, lane 4). A major concern when isolating RNA from blood platelets is contamination with small numbers of leukocytes that contain relatively large amounts of RNA compared with that from platelets. Although the platelet-isolation technique used in this study produced platelet preparations with no apparent leukocyte contamination as assessed by cell counting and flow cytometry analysis, some leukocyte contamination could not be ruled out. We therefore used 2 different approaches to...
investigate the potential influence of leukocyte contamination. First, amplification of platelet cDNA with primers for leukocyte-specific antigens, such as CD2 and CD20, resulted in no amplification products (Figure 4A, lanes 5 and 6). Second, RNA isolated from blood leukocytes, usually discarded during platelet preparation, showed that no TF mRNA was present (Figure 4A, lane 9). These data indicate that platelets were the source of TF mRNA and protein in our assays. To quantify the TF mRNA present in platelets, real-time PCR experiments were carried out. Our platelet preparations contained 0.54 ± 10^−14 pg TF mRNA/ng total RNA, an amount that is >30 times less than the amount of TF mRNA present in TNF-α-activated HUVECs used as an internal control (Figure 4B).

Pretreatment of platelets with the translational inhibitor puromycin slightly reduced (~20%) ADP-induced irTF expression, as assessed by flow cytometry. By contrast, staining of parallel samples for P-selectin was unchanged, suggesting that this inhibitor does not alter the detection of resident proteins (data not shown).

**Effect of Antiplatelet Drugs on Platelet irTF**

Incubation in the presence of iloprost for 15 minutes before ADP stimulation completely abolished irTF expression on platelet membranes (interaction term F = 15.3, P = 0.0009). In contrast, preincubation with aspirin for 15 minutes did not show any effect on irTF expression (F = 0.32, P = 0.6; Figure 5A). Accordingly, iloprost completely suppressed P-selectin expression, whereas aspirin had not such effect (data not shown).

Antagonists of GPIIb/IIIa, at concentrations able to exert 100% receptor occupancy (not shown), increased irTF expression in ADP-stimulated platelets in WB, and this effect was maintained at the highest concentrations used (mean ± SE effect of eptifibatide, +72.8 ± 9.2%, F = 133.9, P < 0.0001; of abciximab, +33.7 ± 7.6%, F = 19.9, P = 0.0003; and of tirofiban, +41.6 ± 9.0%, F = 21.14, P < 0.0002; Fig. 5B). Similar effects were observed in PRP (not shown). Interestingly, only eptifibatide and tirofiban significantly increased irTF expression in unstimulated platelets (+33.7 ± 6.6%, P < 0.0001, and +21.0 ± 6.5%, P = 0.004, respectively; Figure 5B).

To investigate the involvement of GPIIIb/IIia in this paradoxical effect, experiments were performed in platelets from 2 patients with Glanzmann’s thrombasthenia. Results indicated that ADP-induced TF expression in platelets from Glanzmann patients was comparable to that found in platelets from healthy subjects; by contrast, the effect of eptifibatide was completely absent in these patients (Figure 5C), thus suggesting that the GPIIIb/IIia receptor was responsible for TF upregulation induced by GPIIIb/IIia antagonists.

**Discussion**

In this study, we have shown that resting platelets do contain TF protein as well as detectable amounts of TF mRNA. On incubation of platelets with ADP, TRAP, or epinephrine, a substantial increase in potentially active TF was detected on cell membranes. We also have shown that commonly used antiplatelets drugs differently influence the exposure of TF on platelet membranes. Short-term in vitro preincubation of platelets with aspirin failed to influence irTF exposure in-
duced by ADP. Incomplete acetylation of platelet cyclooxygenase or independence from cyclooxygenase-mediated pathways might likely explain this finding. By contrast, the stable prostacyclin analogue iloprost, which markedly increases intraplatelet cAMP levels, completely prevented membrane TF exposure, thus suggesting that the event might be dependent on this biochemical pathway. Interestingly, antagonists of the platelet GPIIb/IIIa tirofiban, epifibatide, and abciximab had no inhibitory effect, but rather epifibatide and tirofiban significantly increased iTF expression in unstimulated platelets. Moreover, epifibatide and abciximab potentiated the stimulatory effect of ADP on platelet iTF. Experiments performed on platelets from Glanzmann’s thrombasthenia patients indicated that this effect was mediated by GPIIb/IIa integrin.

This paradoxical effect is in agreement with the observation of a partial agonist activity of these drugs owing to the increase in “platelet-outside-in signaling,” which leads to increased platelet activation. It was recently reported that GPIIb/IIIa antagonists might cause a conformational change within the GPIIb/IIIa complex, which can be detected by using ligand-induced binding site antibodies, and that ligand-induced binding site epitopes expression might be associated with the partial agonist activity of some GPIIb/IIIa antagonists. The possibility that this mechanism sustains the increase in platelet TF expression and that this might have clinical relevance in vivo as well is intriguing but needs further confirmation.

Platelets, despite the absence of a nucleus, retain mRNAs derived from nucleated megakaryocytes; these mRNAs remain intact throughout the lifespan of the cell and might also be translated into proteins. Our data show that platelets contain a significant amount of TF mRNA, which might potentially contribute to protein synthesis. In our experimental conditions, however, intraplatelet TF synthesis does not significantly contribute to the amount of protein observed on platelet membranes because puromycin did not inhibit TF exposure. We cannot rule out, however, that TF mRNA was translated into protein after several hours of platelet stimulation, as observed by others for interleukin-1β. The translocation of TF from leukocytes to platelets through a CD15-mediated process has been shown by Rauch et al in well-controlled experimental conditions and might be an important mechanism for TF uptake and storage into platelets. However, we were unable, using different approaches, to reveal the presence of leukocytes in our carefully prepared samples of PRP.

Platelets, despite the presence of a nucleus, retain mRNAs derived from nucleated megakaryocytes; these mRNAs remain intact throughout the lifespan of the cell and might also be translated into proteins. This finding suggests a potential role of platelet-derived mRNAs in the regulation of TF expression in vivo.

In conclusion, these data provide an additional explanation for the thrombogenicity of the residual mural thrombus frequently observed in acute coronary syndromes, in which repeated phenomena of thrombosis and dethrombosis take place. Studies in appropriate clinical settings, which are presently ongoing, will define the actual prothrombotic potential of this mechanism.

Acknowledgments
This work was supported by the Italian Ministry of Health RC2002, FIRST 2001. We thank Dr Ivano Eberini for the assistance with hrFVIIa labeling, Dr Fabrizio Veglia for statistical analysis, and Prof Yale Nemerson for his critical reading of the manuscript.

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Arterioscler Thromb Vasc Biol. 2003;23:1690-1696; originally published online July 10, 2003; doi: 10.1161/01.ATV.0000085629.23209.AA
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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**Tissue Factor**

- ADP (10 µM)
  - **WB:** -- ++ +
  - **PRP:** -- ++ +

- TRAP (35 µM)
  - **WB:** -- ++ +
  - **PRP:** -- ++ +

- Epinephrine (1 µM)
  - **WB:** -- ++ +
  - **PRP:** -- ++ +

**P-Selectin**

- ADP (10 µM)
  - **WB:** -- ++ +
  - **PRP:** -- ++ +

- TRAP (35 µM)
  - **WB:** -- ++ +
  - **PRP:** -- ++ +

- Epinephrine (1 µM)
  - **WB:** -- ++ +
  - **PRP:** -- ++ +
Legend to online Figure I

Effect of TRAP and epinephrine on irTF and P-selectin expression on platelet surface. WB and PRP samples were incubated for 15 minutes with buffer or TRAP 35 μmol/L, Epinephrine 1 μmol/L, ADP 10 μmol/L + Epinephrine 1 μmol/L and then analyzed by flow cytometry. *P<0.0001 and #P<0.001 versus control.
Table I. Oligonucleotide primers used for PCR amplification of Reverse-Transcribed RNA.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Oligonucleotide Sequence (5’→3’)</th>
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<tr>
<td>TF forward*</td>
<td>ACTACTGTTCAGTTCAAGCAGTGTGATTC</td>
</tr>
<tr>
<td>TF reverse</td>
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<td>GP1bα forward</td>
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* TF primers were designed to be intron-spanning to rule out amplification of contaminating genomic DNA.