Regular Article

Platelet-associated tissue factor enhances platelet reactivity and thrombin generation in experimental studies *in vitro*

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Introduction

Platelets play a critical role in haemostasis, and are known to contribute to the development of ischemic complications in cardiovascular disease [1]. Upon vessel damage, they recognize and bind to proteins present in the subendothelium to form a platelet aggregate. Simultaneously, TF exposed at sites of vascular damage triggers the coagulation mechanisms to lead thrombin generation, activating other platelets and allowing the formation of a stable fibrin net.

The existence of a circulating source of TF was first speculated and further confirmed within the last decade [2]. This circulating TF has been found associated with certain blood cells, including monocytes, leukocytes, macrophages, platelets [3,4] and cell-derived microparticles [5–7]. A soluble form of TF has been also described [8]. It has been suggested that circulating TF may have a relevant role in thrombus propagation at the site of vascular injury [9].

Since the description of a circulating source of TF in blood, a debate has been generated on whether this source of TF circulates as a soluble form, associated to microparticles or carried by other blood cells including platelets among them. However, the association of TF with platelets has generated some controversy [4,10–12]. Del Conde et al. [11] reported that TF-bearing microvesicles fused with activated platelets, by using dual colour flow cytometry techniques. Two different groups

Abbreviations: TF, tissue factor; TF-MV, human TF-rich microvesicles; pTF, TF-MV from human placenta; rTF, TF-MV from recombinant origin; PRP, platelet-rich plasma; LMWH, low molecular weight heparin; Col, collagen; % CS, percentage of surface covered by platelets vs. the total area screened; % T, percentage of surface covered with thrombus vs. the total area screened; Area T, mean area of thrombin; % F, percentage of surface covered with fibrins vs. the total area screened; CT, clotting time; CFP, clot formation time; A10, clot amplitude after 10 minutes.

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identified TF in resting platelets and reported that activation of platelets under static conditions led to an expression of TF on the platelet surface after secretion [4,10]. Interestingly, activation of platelets led to the expression of TF on the platelet surface and to the release of TF-bearing microparticles. Studies from our group demonstrated that platelets can interact with TF immobilized on a surface [13,14], but also that they possess mechanisms to internalize TF-rich microvesicles (TF-MV) from different sources [15,16]. In the previously referred studies platelets suspensions were exposed in vitro to preparations of TF-MV and vesicles containing TF were internalized through the open canicular system membranes and, occasionally found in the alpha-granules. The previous processes occurred independently of the purity of the TF, though they were accelerated by the presence of residual antigens contaminating the microvesicles [15,16].

Taking into consideration that platelets can avidly interact and internalize circulating pathogens and particulates [17] we hypothesize that small amounts of TF internalized by platelets could play a critical role promoting thrombus formation and thus, contributing to the development of acute thrombotic events. In the present studies we have investigated the in vitro effect of platelet-associated TF in thrombogenesis using different experimental approaches with blood subjected to flow or steady conditions.

Materials and methods

The investigation conforms with the Guide of Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996), with the Directive 2010/63/EU of the European Parliament and with the principles outlined in the Declaration of Helsinki. This study has been approved by our institutional Ethics Committee (2009/4700).

Experimental design

Platelet rich plasma (PRP) was isolated from anticoagulated blood drawn from healthy individuals. Aliquots of PRP were incubated with two different preparations of human TF-MV: placental TF (pTF) and recombinant relipidated TF (rTF), to facilitate the incorporation of TF-MV into platelets, as previously described [15,16,18]. Excess of TF-MV was removed from incubates by repeated washing. Levels of TF associated to platelets were quantified using a coagulometric assay. Adhesive and cohesive properties of platelets containing TF were assessed in perfusion models using two different flow devices and thrombogenic surfaces. Perfusion studies were performed with heparinized blood after addition of 30% of washed platelets exposed to TF-MV. Results were always compared with experiments using 30% of washed platelets that were processed in parallel, but not exposed to TF-MV. Thrombin generation and viscoelastic properties of the forming clot were also assessed. Inhibitory strategies with a specific antibody to TF were performed for some experimental purposes.

Reagents and antibodies

Low molecular weight heparin (LMWH) was from Fragmin®, Pharmacia & Upjohn, Sweden. Fibrillar collagen type I was purchased from CHRONO-LOG Corporation (USA). Phosphate buffered saline (PBS) was from Gibco BRL Life Technologies (Paisley, UK). Monoclonal antibody against human tissue factor (No.4509) was from American Diagnostica inc (Stamford, USA). The embedding kit JB-4 for histologica processing was from Polyscience (Warrington, USA). KOTEM Thromboelastometry reagents were from PentapharmGmbH (Munchen, Germany). Thrombin generation was assessed with the fluorogenic assay Technothrombin ® TGA RA/MFC from Technoclone GmbH (Vienna, Austria).

Tissue factor sources

TF-MV from human placenta (pTF) (Thromborel®S, Siemens Healthcare (Marburg BmbH, Germany)) and human recombinant relipidated TF (rTF) (Innovin®DADE®, Siemens Healthcare (Marburg BmbH, Germany)) were reconstituted according to the manufacturer’s instructions to reach final concentrations equivalent to 1.1 nM for the pTF, and 0.9 nM for the rTF. Briefly, the pTF preparation consists of microvesicles heterogeneous in size that besides TF exposes other concomitant antigens such as CD14, CD45 and CD62-P [15]. The rTF preparation consists of smaller microvesicles more homogeneous in size of relipidated recombinant human TF and does not expose other antigens. Both TF-MV preparations proved to be internalized by platelets, and the presence of TF in these microvesicles was already confirmed by immunocytochemical and flow cytometry techniques [15].

Preparation of platelet suspensions containing tissue factor-rich microvesicles

Blood was drawn from healthy donors who had not taken any drug known to affect platelet function in the previous 10 days. Blood samples were anticoagulated with citrate/phosphate/dextrose (CPD) at a final citrate concentration of 19 mM. Platelet rich plasma (PRP) was obtained by centrifugation at 120 × g for 15 min. PRP aliquots were incubated with buffer saline (control sample), or with TF-MV (0.11 nM pTF and 0.09 nM rTF, final concentrations), for 30 min to allow optimal internalization [15]. After incubation with the TF-MV preparations, platelets were washed three times with citrate/citric acid/dextrose (93 mM sodium citrate, 7 mM citric acid and 140 mM dextrose, pH 6.5, containing 5 mM adenosine and 3 mM theophylline) to remove the excess of TF and obtain the TF-enriched platelet suspensions.

For thrombin generation assays, the final platelet pellet was resuspended in the microparticle free plasma supplied by the Technothrombin® TGA RA/MFC kit. For the other experimental approaches the final pellet of platelets was resuspended in HBSS supplemented with dextrose and NaHCO₃ (pH 7.2), and rested for 45 min at 37 °C before being used.

Quantification of tissue factor associated by platelets

Evaluation of TF tissue factor associated to platelets was assessed through a two-stage clotting assay applied to platelets lysates. Platelets incubated with buffer saline were used as negative control. The final platelet count was 200 × 10⁵ platelets per microliter. Platelet suspensions were lysated by freeze-thaw cycles and further ultracentrifuged (600000 ×g, 10 min, 10 °C) in a Sorvall MTX 150 Micro-Ultracentrifuge (Thermo Scientific; Japan), to isolate the TF-bearing microvesicles. TF quantification was performed using a two-stage clotting assay as previously described [6,19]. The clotting end-point was determined in a Dade Behring BPT® II Analyzer (Marburg BmbH, Germany). The final amount of TF was achieved by interpolating the clotting time into a standard curve obtained with dilutions of a recombinant relipidated TF (Innovin®DADE®). The effect of an antibody to TF was also investigated in these samples. TF preparations (pTF and rTF) were incubated with the anti-TF (10 μg/ml) for 1 h at room temperature. Next, this TF was added to the PRP aliquots (1/10) and further incubated for another hour at 37 °C. Afterwards, platelets were washed to remove excess of TF-MV as explained above. Concentrations are expressed as picomolar TF in 200 × 10⁵ platelets per microliter lysates.

Studies with flowing blood

Adhesive and cohesive properties of platelets containing TF-MV were assessed in perfusion models. Aliquots of washed TF-enriched platelets,
prepared as described above, were added to blood anticoagulated with LMWH (20 U/ml), to increase by 30% the initial platelet count. Platelets incubated with buffer saline were used as negative control.

Perfusion studies were performed using two different devices: a flat perfusion chamber that uses small volumes of blood per run and an annular perfusion chamber. The inhibitory effect of an antibody against TF was investigated in the flat chamber device.

Interaction of platelets with collagen in flat chambers

Less than one ml of reconstituted blood was perfused through a flat microchamber using immobilized type I collagen (Col) as thrombogenic substrate [14]. Col was sprayed to achieve a coat concentration equivalent to 30.9 μg/cm². Perfusions were performed at a shear rate of 600 s⁻¹, for 5 min. After perfusion, surfaces were rinsed with PBS (0.15 M), fixed with 0.5% glutaraldehyde (in 0.15 M PBS) at 4 °C for 24 h and stained with toluidine blue 0.05% for morphometric en-face evaluation. Images from the perfused surfaces were obtained using a digital camera (ProgRes® MF Cool camera and ProgRes® Capture Pro 2.0 software, Jenoptik, Germany) adapted to a light microscope (Polyvar Widefield Photomicroscope, Reichert-Jung, Austria). A software package (Sigma Scan Image Analysis Software, version 4.00.023, from Jandel Scientific) provided specialized programs to determine the gray level histogram of each image [20]. This program determines the percentage of surface covered by platelets (% CS) versus the total area screened.

Interaction of platelets with vascular subendothelium in annular chambers

Twenty-two ml of reconstituted blood were perfused in an annular chamber using damaged vascular segments from rabbit aorta, as thrombogenic substrate. Aortas were extracted from young female New Zealand rabbits (2.8-3.0 Kg). Rabbits were previously euthanized using an overdose of anaesthetic (sodium pentobarbital; 500 mg/Kg). Vessels were cleaned, everted, cut into segments and euthanized using an overdose of anaesthetic (sodium pentobarbital; 500 mg/Kg). Vessels were cleaned, everted, cut into segments and maintained in PBS. The endothelial layer was chemically removed with α-chymotrypsin to expose the subendothelial layer. Vascular segments were stored in PBS at −20 °C until used [21]. Perfusions studies were performed at a shear rate of 600 s⁻¹, for 10 min. After perfusion, vascular vessels were rinsed with PBS (0.15 M), fixed with 2.5% glutaraldehyde (in 0.15 M PBS) at 4 °C for 24 h and processed histologically for further morphometric evaluation. Fibrin deposition and platelet interaction were evaluated by a light microscope connected to a computer, provided with a special software, that automatically classifies and quantifies platelet and fibrin coverage [22]. Platelet interaction was expressed as a percentage of covered surface by platelets (% CS) and as the mean area of platelet cross-sectional interaction (Area P; μm²). Furthermore, using a specially developed computer program, platelets or groups of platelets were classified as: contact, platelets attached but not spread on the subendothelium; adhesion, platelets which had spread on the subendothelium surface including small aggregates of less than 5 μm in height; and thrombi, large platelet aggregates of more than 5 μm in height. Similarly, the presence of fibrin was also morphometrically quantified and expressed as a percentage of fibrin.

Thromboelastometry studies

In order to evaluate the influence of TF-enriched platelets on the whole blood clot formation, we investigated the dynamic thrombelastography of whole blood coagulation, using the ROTEM Thromboelastometry Analyser (PentapharmGmbH, Munich, Germany) [22]. Platelets were added to blood anticoagulated with LMWH (20 U/ml), to increase by 30% the initial platelet count. Blood reconstituted with platelets not exposed to TF-MV was used as negative control. The technique was performed according to the manufacturer’s instructions. We used the commercial test EXTEM that measures changes on the extrinsic pathway of coagulation, fibrinogen and fibrin polymerization, and platelet function. Clots obtained in the EXTEM are composed of platelets and fibrin. This analyser provides several parameters. We have focused in the clotting time (CT) and the clot formation time (CFT), which indicate the dynamics of clot formation; and in the clot amplitude that gives information about clot strength and stability, which is largely dependent on fibrinogen and platelets. The CT is defined as the time when the forming clot reaches 2 mm; the CFT is the time when this clot reaches 20 mm; and the clot amplitude after 10 minutes (A10) would be a measure of clot firmness.

Attending to our experimental design, some modifications of the standard technique were included. Experiments were performed adding the isolated platelets to blood anticoagulated with LMWH (20 U/ml) from the same donor, to increase by 30% the initial platelet count. Profiles of continuous whole blood clot formation were recorded for 30 minutes. Experiments using a neutralizing anti-TF antibody were also performed. PRP aliquots were exposed (1/10) to TF preparations (pTF and rTF) that had been previously incubated with the anti-TF (10 μg/ml) for 1 h at room temperature, and further incubated for another hour at 37 °C. Unbound TF-MV (± anti-TF) were removed by washing platelets as explained above, to obtain a TF-rich platelet suspension. A control with saline buffer incubated with anti-TF was also performed to detect the effect of residual amounts of the anti-TF.

Thrombin generation assay

Thrombin generation on citrated PRP was assessed with a fluorogenic assay as previously described [18]. This assay is based on the fluorescence generated by the cleavage of a fluorogenic substrate by thrombin over time upon activation of the coagulation cascade by TF and negatively charged phospholipids in plasma. In our experimental setting we used the TGA reagent A, a TF free reagent consisting in low concentration of phospholipids micelles, so that the only TF present is the endogenous on our samples. For these experiments, washed platelets were reconstituted in microparticle free plasma reaching a final platelet concentration of 200 × 10³ platelets per microliter. We evaluated thrombin generation in PRP samples with those platelets containing pTF and rTF. Platelets incubated with buffer saline were used as negative control. An inhibitory strategy using an antibody against TF was also applied. TF preparations (pTF and rTF) were incubated with the anti-TF (10 μg/ml) for 1 h at room temperature, and then TF was added to the PRP aliquots (1/10) and further incubated for another hour at 37 °C. Afterwards, platelets were washed to remove excess of TF-MV and the final pellet resuspended in the microparticle free plasma provided by the manufacturer to generate a PRP in which the only TF present is the platelet-associated tissue factor. A control with saline buffer incubated with anti-TF was also performed to detect the effect of residual amounts of the anti-TF.

Brieﬂy, duplicated PRP aliquots of 40 μl were inserted into a fluorescence plate, mixed with 50 μl of a reagent with the anionic phospholipids; and with 10 μl of the fluorogenic substrate in calcium that triggers the assay. Fluorescence generated was measured at 1 min intervals for 90 min at a wavelength of 390 nm / 450 nm (excitation / emission). The evaluation program, Technothrombin® TGA for Thermo Fluoroskan Ascent Software (version 2.6) facilitated by Technoclone GmbH (Vienna, Austria), transforms the relative units of fluorescence into thrombin concentration (nM) and provides the time to achieve this peak of thrombin (min). Results are expressed as Mean ± SEM.

Statistics

Results were expressed as mean ± standard error of the mean (SEM). One way ANOVA test for independent experiments was applied when multiple comparisons were required. Student’s t-test
for paired data was used for comparisons in perfusion experiments. The level of statistical significance was established at least at \( p < 0.05 \), when statistical significance reached \( p < 0.01 \) it was pointed out.

**Results**

**Coagulometric assessment of platelet-associated tissue factor**

TF-procoagulant activities associated to platelet lysates were determined in suspensions of \( 200 \times 10^3 \) platelets per microliter. The average amount of procoagulant TF was 2.0-fold higher in washed platelets previously exposed to TF-MV than in control platelets (\( p < 0.05 \) vs CON). Previous incubation of TF-MV with an antibody to TF, resulted in clotting times similar than those observed in the control conditions. Results for each preparation of TF-MV, pTF and rTF, with and without anti-TF, are summarized in Table 1. No significant differences were observed for the two different TF preparations.

**Thrombogenic action of platelets containing tissue factor**

The mean platelet count in LMWH whole blood was \( 177.4 \pm 8.7 \times 10^3 \) platelets per microliter (\( n = 9 \)), and the final platelet count upon enrichment with a 30% of platelets either control or exposed to TF-MV was \( 229.1 \pm 10.8 \times 10^3 \) platelets per microliter.

**Interaction of platelets with Col in flat chambers**

Covered surface by platelets on perfused coverslips coated with Col was \( 20.6 \pm 2.0\% \) for blood samples containing a 30% of control platelets. The same experiments with platelets containing pTF or rTF, revealed a statistically significant increase in the platelet coverage (% CS) vs controls, in all cases, with % CS of \( 26.2 \pm 2.2 \) and \( 27.9 \pm 1.7 \) respectively (\( p < 0.01 \)). Moreover, the platelet coverage appeared statistically augmented (\( p < 0.05 \)) in those perfusates with rTF versus those containing pTF. Results are summarized in Fig. 1. The inhibitory action of the antibody to TF was similar for both preparations of TF-MV (pTF or rTF) with % CS returning to baseline levels observed in control experiments (\( 21.7 \pm 2.3 \) and \( 21.0 \pm 2.1 \) for pTF and rTF with anti-TF, respectively).

**Interaction of platelets with vascular subendothelium**

Results from perfusion studies in annular devices match those observed in flat chambers. The presence of platelets containing pTF or rTF increased the surface covered by platelets on the subendothelium vs controls (% CS: \( 54.0 \pm 1.5 \) and \( 47.2 \pm 6.8 \) respectively vs. \( 38.0 \pm 3.5 \), \( p < 0.05 \)). As shown in Fig. 2, larger platelet aggregates were observed when platelets containing TF were present in the perfusates. This was confirmed in the morphometric analysis with significant increases in both, the percentage of surface covered with large aggregates (%T) and the mean area of those aggregates (in \( \mu \text{m}^2 \)), as summarized in Table 2. Effects of platelet-associated TF on fibrin formation were less evident, with moderate increases that did not reach the levels of statistical significance.

**Thromboelastometry studies**

Thromboelastometry studies using LMWH blood aliquots enriched with 30% of control platelets or platelets containing pTF or rTF, showed some statistical significant reductions (\( p < 0.05 \)) on the dynamics of clot formation parameters (CT and CFT) in samples containing TF-enriched platelets when compared to the control samples, not exposed to TF. However, the clot firmness assessed as A10 values was only slightly increased with respect to the control experiments, but differences never reached the levels of statistical significance. These findings were consistent and of a similar intensity for both, pTF and rTF preparations.

The use of an antibody to TF partially prevented the reductions in the CT by platelet associated pTF and rTF, with inhibitions of 20.5% and 25.2% respectively. Also a delay in the CFT was measured for both samples containing pTF and rTF with elevations in the equivalent to 12.9% and 25.4%, respectively. No effects were detected in the control samples with anti-TF.

Results of these experiments are summarized in Fig. 3.

**Modifications in thrombin generation**

As shown in Fig. 4, the presence of TF-enriched platelets accelerated thrombin generation confirmed by a significant shortening in both, time to start thrombin generation (lag phase) and time to reach the maximal thrombin peak with respect to control platelets (\( p < 0.001 \)). However, the maximal peak of thrombin generated was only significantly increased for platelets containing pTF (\( 619.2 \pm 14.8 \) nM vs. \( 407.3 \pm 21.4 \) nM in the respective control samples; \( p < 0.001 \)), but not for those containing rTF. The inhibitory strategy using a neutralizing antibody to TF showed a statistical significant delay in both the lag phase and the time for maximal thrombin peak, thus partially preventing the effects observed with samples containing TF-MV but not for the control samples with the antibody. The inhibitory action of the antibody to TF also lowered to \( 474.69 \pm 5.21 \) nM the thrombin peak obtained in samples containing pTF (\( p < 0.001 \) vs. pH).

**Discussion**

In the present study we have investigated the possible thrombogenic role of platelet-associated TF. Data raised form our experimental studies confirm that the presence of platelets containing TF potentiated adhesive and aggregating properties of platelets on thrombogenic surfaces, at elevated shear rates, and improved viscoelastic properties of clots formed, under lower shear conditions. These findings were independent on the purity of the human TF-MV preparation, being similarly observed with TF from placental origin (pTF) or with recombinant relipidated TF (rTF). The mechanism of the thrombogenic action of TF associated to platelets could be explained through a faster and enhanced local thrombin generation.

Since the description of a circulating source of TF, a debate has been generated on whether this new source of TF circulates as a soluble form, associated to microparticles or carried by platelets. While the prothrombotic role of soluble TF has not been confirmed, several reports have demonstrated increased levels of circulating TF-rich microparticles in patients with cardiovascular risk factors such as hypertension [23], diabetes [24,25], metabolic syndrome [25]; dyslipidemia [26], smoking habit [26]; and acute coronary syndrome [27,28]. The pharmacological treatment or control of the previous pathological conditions seems to result in a reduction of the circulating levels of TF [22,26,29,30]. Furthermore, there are pathological conditions in which it has been reported an increased number of TF-positive platelets such as essential thrombocythemia, polycythemia vera, cancer, acute coronary syndrome [31–34]. Due to all these evidence, the presence of circulating TF has been suggested as candidate biomarker with predictive value for future cardiovascular events. It is worth mentioning, that inflammatory
conditions have been implied in the development of acute events in cardiovascular pathologies, leading to the generation of TF-rich microparticles [35] that could be freely transported through the circulation internalized by platelets [15]. It has also been suggested that inflammatory conditions could induce de novo synthesis of TF by platelets [36]. A question that remains unsolved is whether the internalization of TF-MV by platelets is a mechanism to sequester and inactivate microparticles expressing TF or contrarily platelets could still deliver the stored TF into a growing thrombi.

Interest has been raised about the presence and pathological implications of TF associated to platelets [37]. It has been postulated that circulating TF may have a relevant role in thrombus propagation being transferred to platelets during thrombus growth [9]. Other groups identified TF in resting platelets and found expression of this TF on the platelet surface after the activation of platelets under static conditions [4,10]. It has been suggested that platelet-TF could be transferred from PMN leukocytes [38]; and that leukocyte CD15 and platelet CD62-P would be involved in such process [39]. Studies from our group demonstrated that platelets possess mechanisms to internalize and store TF-MV and that CD62-P could favor this internalization process [15]. Despite the previous reports, considerable debate exists concerning the presence and clinical relevance of platelets associated TF [12,40–42].

In our present experimental studies we have confirmed that TF-MV associates to platelets under our experimental conditions. Coagulometric assays revealed a 2.0-fold increase in the TF-related procoagulant activity in these platelets versus control platelets. Previous ultrastructural studies applied to the platelet suspensions exposed to TF-MV preparations, revealed that around 10–25% of platelets contained TF-MV (data not shown). Based on these calculations, the final proportion of platelets containing TF-MV in the perfusates could range from 3 to 10%. Despite the presumably lower proportion of TF-enriched platelets in the perfusates, our studies with circulating blood demonstrated enhanced platelet-aggregate formation on both, vascular and Col-coated surfaces. These findings substantiate the hypothesis that platelets containing TF are more reactive to thrombogenic surfaces, thus promoting an increase in their adhesive and aggregating properties. Giesen and cols [2] observed an increased fibrin generation after perfusing native blood over collagen-coated slides that was markedly reduced in the presence of a potent inhibitor of TF. In our experimental settings, under flow conditions similar to those used in the previous report, we were able to detect enhanced platelet recruitment into aggregates by incorporating a relatively low amount of platelet-associated TF. Interestingly, incubation of TF-MV with a TF antibody prevented this enhancement resulting in patterns of platelets interaction similar to those observed in control experiments with platelets not exposed to TF-MV.

Although arterial and venous thromboembolic disorders are usually considered as two distinct entities, recent evidence seems to indicate
Table 2
Effect of platelet-associated tissue factor (TF) on the platelet coverage (% CS) and on the large aggregates formation (T) (percentage of coverage and mean area of large aggregates), onto damaged vessels, under flow conditions.

<table>
<thead>
<tr>
<th></th>
<th>control</th>
<th>pTF</th>
<th>rTF</th>
</tr>
</thead>
<tbody>
<tr>
<td>CS (%)</td>
<td>38.0 ± 3.5</td>
<td>54.0 ± 1.5$^*$</td>
<td>47.2 ± 6.8$^*$</td>
</tr>
<tr>
<td>T (%)</td>
<td>23.6 ± 2.1</td>
<td>39.4 ± 4.7$^*$</td>
<td>35.2 ± 6.2</td>
</tr>
<tr>
<td>Area T (μm²)</td>
<td>121.1 ± 6.3</td>
<td>183.0 ± 33.3</td>
<td>166.8 ± 10.0$^*$</td>
</tr>
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Mean ± SEM (n = 9).
pTF: placental human tissue factor.
rTF: recombinant relipidated human tissue factor.
$^*$ p < 0.05 vs. control.

thrombin has also been shown, linking the platelet phenotype to the thrombotic complications of the diseases. As far as the acute thrombotic event in cardiovascular disease is concerned, Brambilla and cols [32] reported that patients with acute coronary syndrome have a significantly higher amount of TF-positive platelets compared to patients with stable angina or controls and this is paralleled by a significantly higher thrombin generation capacity. Our present experimental data would also indicate that platelet-associated TF can also enhance and accelerate blood clotting under reduced flow conditions. The mechanism by which platelet associated TF exerts an increased thrombogenic response under arterial and venous experimental conditions could be explained by the faster and enhanced local thrombin generation favouring further platelet activation and contributing to thrombus propagation. Our experimental studies cannot answer whether platelets transporting TF-MV will remain, or be rapidly removed from the circulation in patients. Clinical studies should be designed to investigate this possibility in selected populations of patients exposed to elevated cardiovascular risks.

In summary, our experimental data indicate that TF-associated to platelets would enhance platelet thrombus formation at damaged vascular sites contributing to arterial complications, but also prime the dynamics of blood clot formation under venous flow conditions. An enhanced thrombin generation could be the mechanism implied in the potentiation of thrombus formation observed in our studies. Further studies are still required to confirm the role of TF associated to platelets in the development of thrombotic complications in patients and also on the possibility to design inhibitory strategies to prevent TF uptake by platelets.
Conflict of interest statement

There are no conflicts of interest to declare.

Author Contributions

- I. Lopez-Vilchez: design of the study, acquisition and analysis of data, writing the manuscript.
- A. M. Galan: concept and design of the study, acquisition and interpretation of data, writing the manuscript.
- M. R. Hernandez: Acquisition, analysis and interpretation of data.
- C. Caballo: Acquisition, analysis and interpretation of data.
- M. Roque: writing the manuscript and revising the intellectual content.
- M. Diaz-Ricart: concept of the study, revising the intellectual content.
- J. G. White: interpretation of ultrastructural data and revising the intellectual content.
- G. Escolar: concept and design of the study, interpretation of data and writing the manuscript.

All authors have approved the final version to be submitted

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References