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The Role of Tissue Factor in Atherothrombosis and Coronary Artery Disease: Insights into Platelet Tissue Factor

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Semin Thromb Hemost

Abstract

The contribution of vessel wall-derived tissue factor (TF) to atherothrombosis is well established, whereas the pathophysiological relevance of the blood-borne TF is still a matter of debate, and controversies on the presence of platelet-associated TF still exist. In the past 15 years, several studies have documented the presence of TF in human platelets, the capacity of human platelets to use TF mRNA to make de novo protein synthesis, and the increase in the percentage of TF positive platelets in pathological conditions such as coronary artery disease (CAD). The exposure of vessel wall-derived TF at the site of vascular injury would play its main role in the initiation phase, whereas the blood-borne TF carried by platelets would be involved in the propagation phase of thrombus formation. More recent data indicate that megakaryocytes are committed to release into the bloodstream a well-defined number of TF-carrying platelets, which represents only a fraction of the whole platelet population. These findings are in line with the evidence that platelets are heterogeneous in their functions and only a subset of them is involved in the hemostatic process. In this review we summarize the existing knowledge on platelet associated TF and speculate on its relevance to physiology and to atherothrombosis and CAD.

Keywords
- ► tissue factor
- ► platelets
- ► megakaryocytes
- ► atherothrombosis
- ► coronary artery disease

The publication of studies undertaken in the last century to characterize the role of tissue factor (TF) in physiological and pathological processes has seen a sharp increase since 1987, when the gene coding for human TF was sequenced and the primary structure of the TF apoprotein deduced.[1–4] Since then, and until the beginning of the new century, the contribution of TF to the atherothrombotic process was assessed focusing on the vessel wall-derived TF, the only source of TF believed at the time to be present in the human body. Then a new player came onto the scene, the blood-borne TF, a term originally introduced to allude mainly to circulating microvesicles (MVs)-carrying TF, which can be taken up by circulating platelets.[5,6] This term now refers to TF present in the circulation as a whole, whether it is derived from MVs, or from activated leukocytes or platelets.

The findings that have established the role of vessel wall-derived TF in atherothrombosis have been revised in several reviews, which the reader is referred to.[7–12] By contrast, the pathophysiological relevance of the blood-borne TF is still a matter of debate, and controversies on the presence of

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platelet-associated TF still exists.[13–17] In this review, we aim to summarize the existing knowledge on platelet-associated TF and to speculate on its relevance to physiology and to atherothrombosis and coronary artery disease (CAD).

From Blood-Borne Tissue Factor to Platelet-Associated Tissue Factor in Humans

Historical Background

Toward the end of the last century several studies provided evidence for the presence of detectable blood-borne TF activity in healthy subjects[18–20] and for increased levels of circulating TF antigen in patients suffering from cardiovascular disease,[21–23] sepsis,[24] hematologic disorders,[19,25] and disseminated intravascular coagulation.[24,26] As a result, an increased blood thrombogenicity in those disease states was also described.[27] At the same time, the first articles reporting the presence of procoagulant MVs in the circulating blood of humans under normal and pathophysiological conditions appeared in the literature.[5,28–32] The presence of "TF–platelet hybrids" were first discovered and characterized in Yale Nemerson's laboratory by Giesen et al in 1999 and Rauch and Nemerson in 2000: circulating platelets, carrying TF through the fusion of TF-containing MVs with the membrane of activated platelets through a CD15-P-selectin transfer mechanism, may themselves trigger the activation of the blood coagulation cascade.[5,33] Based on these new findings they also proposed a revised mechanism of thrombus formation foreseeing a concerted action of the two different pools of TF: by this mechanism the exposure of vessel wall-derived TF at the site of vascular injury would play its main role in the initiation phase, whereas the blood-borne TF carried by platelets would be involved in the propagation phase of thrombus formation.[5,6,33] The Nemerson group based the need for an extra source of TF in thrombus formation on the assumption that, as soon as platelets start to accumulate at the site of injury, they hamper the interaction of the vessel wall-derived TF with FVII and the activation of FIX and FX, thus affecting the generation of the prothrombinase activity on the surface of a growing thrombus.[34] In this scenario, the platelet-associated TF may therefore sustain the propagation phase of thrombus formation.

Since then, several articles have reported the presence of TF in human platelets. In 2001, Zillman et al observed by flow cytometry that a short (5 minutes) stimulation of whole blood with collagen resulted in an increase of the quantity of TF antigen associated with monocytes and granulocytes.[35] The authors observed that when the amount of platelets in whole blood was gradually reduced, the amount of TF on leukocytes also decreased. Moreover, the increase in TF antigen elicited by collagen was exclusively noted in the CD41a-positive leukocytes, suggesting therefore that platelets are a source of TF. It should be mentioned in this regard that nowadays we are able to provide the demonstration of this hypothesis taking advantage from the latest generation flow cytometers developed in recent years, which combine the fluorescence sensitivity of flow cytometry with the functional insights of high-resolution microscopy. Imaging flow cytometry allows us to characterize with absolute precision the cell type that expresses TF. Taking advantage from these tools it is possible to confer TF origin to one cell or another, especially when leukocyte–platelet aggregates are studied, performing a whole blood flow cytometry analysis, a method characterized by minimal sample handling (►**Fig. 1**). In the following years, we and other investigators identified TF antigen in human platelets from healthy subjects by using, besides flow cytometry, several other methodological approach including western blotting, enzyme-linked immunosorbent assays, confocal, and electron microscopy.[36–38] The most relevant finding of these studies was the observation that stimulation of platelets with agonists such as adenosine diphosphate (ADP), thrombin, epinephrine, thromboxane analogue U46619, and calcium ionophore A23187 resulted in the expression of TF, together with other common markers of platelet activation such as P-selectin or activated glycoprotein IIb/IIIa (GpIIb/IIIa), on the platelet membrane. This event was time-dependent with the maximal expression of TF observed 15 to 30 minutes after stimulation and was also concentration-dependent.[14,15,36,37] Moreover, in our experimental setting, we also showed that the platelet-associated TF was functionally active, being able to bind to FVIIa and to trigger FXa and thrombin generation.[37,39] We also provided the evidence, for the first time, that platelets contain the TF mRNA.[37,39] This finding was criticized at the beginning due to the failure, by other investigators, to get the same results.[35,40] Indeed, the presence of TF mRNA in platelets was seen as the result of a leukocyte contamination during RNA preparation. A few years later, two independent groups not only confirmed our finding, but they also showed that platelets, upon stimulation, can use the TF mRNA to make de novo protein synthesis.[41,42]

Since both platelets and TF are involved in the etiopathogenesis of many diseases, studies were performed to assess the expression of platelet-associated TF in pathological conditions such as coronary artery disease, diabetes, essential thrombocythemia, and cancer.[39,43–45]

In the setting of CAD we showed that the number of circulating TF-positive platelets and TF-positive platelet–monocyte aggregates in patients with acute coronary syndrome (ACS) was significantly higher than those found in patients with stable angina and in healthy subjects.[39] Moreover, we reported that the number of TF-positive platelets was not only three times higher in ACS, but each platelet expressed twice the number of TF molecules than in the two other groups studied, so that the total capacity to generate thrombin was greater. Thus, these findings suggest from one side that TF-bearing platelets as well as platelet-leukocyte aggregates may contribute to thrombus formation upon plaque rupture providing a further explanation of the increased cell thrombogenicity documented in ACS; from the other side, TF-bearing platelets as well as platelet–leukocyte aggregates might also be responsible of thrombus generation in distal sites.

Moreover, the levels of TF mRNA in platelets from ACS patients were also higher compared with those found in patients with stable angina and in healthy subjects. This

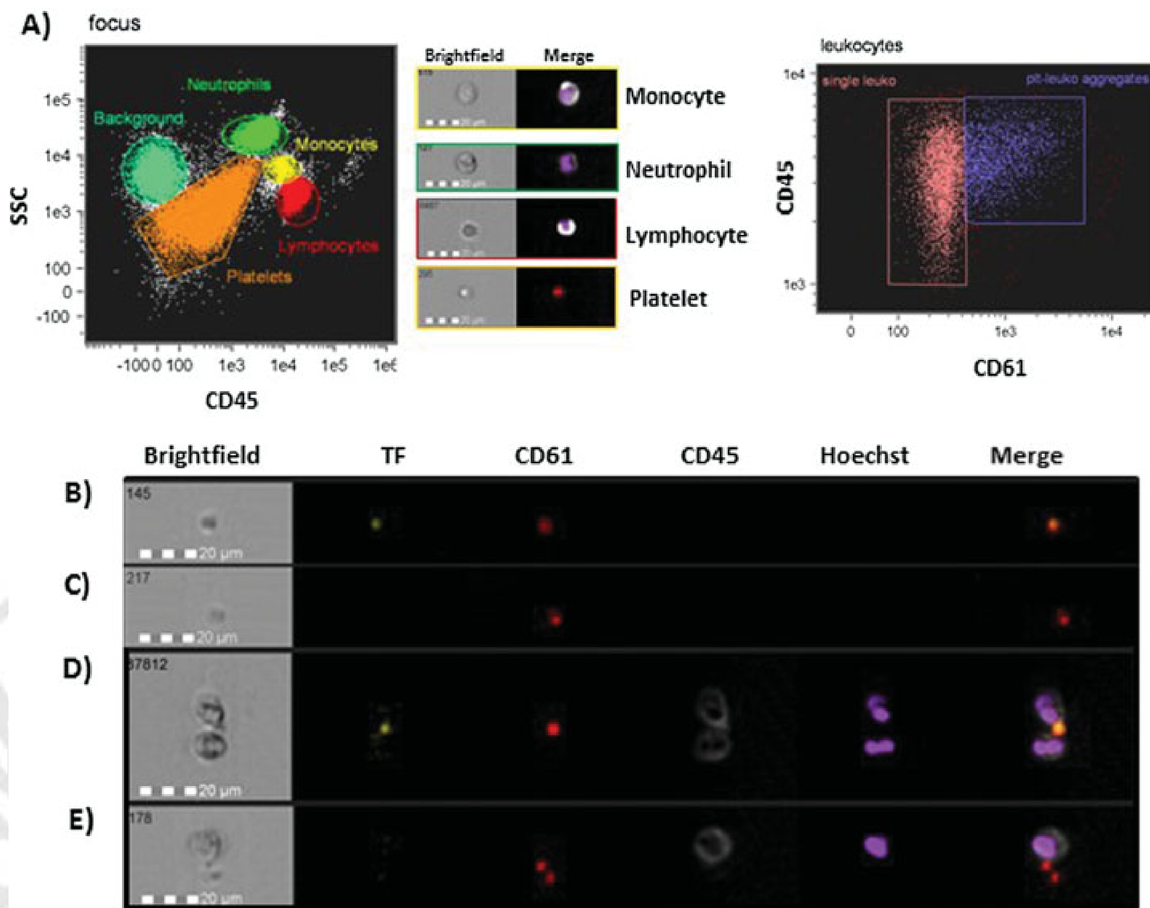

Fig. 1 Identification of tissue factor (TF)-positive platelets by imaging flow cytometry. Stimulation of whole blood with ADP (10 μM, 15 minutes) results in an increased expression of platelet- and platelet–leukocyte aggregates (PLA)-associated TF. Single-cell analysis, performed on high-resolution images of each cell in flow by imaging flow cytometry (FlowSight; Amnis, EMD Millipore, Billerica, MA), indicates that TF expression in PLA is localized exclusively on platelets. A representative scatterplot of whole blood is reported in panel (A). Monocyte, granulocyte, and lymphocyte populations were discriminated according to a physical parameter (side scatter, SSC) and CD45 expression. Platelets and PLA were identified based on CD61 antigen expression. Representative brightfield and CD61 (red)/CD45 (white)/Hoechst (magenta) composite images (merge) are shown. Single cell images, representative of (B) TF-positive platelet; (C) TF-negative platelet; (D) TF-positive PLA; (E) TF-negative PLA are shown. Images were acquired at ×40 magnification. Channel images of Brightfield (gray), TF (green), CD61 (red), CD45 (white), Hoechst (magenta), and the composite image are reported as displayed by the Amnis IDEAS data analysis software. ADP, adenosine diphosphate.

finding is of particular relevance considering the well-described biosynthetic capacity of platelets.[46–48]

Several mechanisms may be responsible for the increased number of TF-positive platelets observed in ACS patients, including a higher amount of TF-positive platelets released by megakaryocytes into the blood stream or de novo synthesis of TF protein in the circulating platelets following splicing of the TF pre-mRNA (see below and ►**Fig. 2**).[39] Appropriate studies however are required to test these hypotheses.

Mechanisms Responsible for the Presence of Tissue Factor in Platelets: The Megakaryocyte-Platelet Axis

Data accumulated over the past 15 years suggest that at least three mechanisms may be involved in the presence of TF in platelets: (1) the MV-transfer mechanism; (2) the storage within the α-granules and the open canalicular system, as reported by Muller et al,[38] and (3) the de novo protein synthesis from the TF-specific messenger RNA.[49] We believe that these pathways are not mutually exclusive and one mechanism may dominate over the other depending on the pathophysiological conditions. Despite this evidence, it is still commonly believed that the only mechanism responsible for the presence of TF in platelets is through the uptake of TF-positive microparticles released by activated endothelial cells or leukocytes. We and others, however, have provided evidence that not only human platelets, but also human megakaryocytes contain TF mRNA.[37,39,41] Therefore, it can be speculated that the TF mRNA and the protein detectable in platelets could be the result not only of a MV-transfer mechanism, but also of a direct transfer from megakaryocytes.[50] To test this hypothesis, we took advantage of an in vitro cell culture model able to recapitulate megakaryocyte differentiation and platelet biogenesis (Meg-01 megakaryoblast cell line), providing consistent evidence that TF is an endogenously synthesized protein that characterizes megakaryocyte maturation. This in vitro approach allowed us to study mRNA and protein expression in platelets in the absence of any cross-talk with other cells or MVs. Indeed,

in these experimental conditions we showed the existence of a direct transfer of both TF pre-mRNA and protein from megakaryocytes to a subset of platelets where it contributes to their thrombin generation capacity. Of interest, the percentage of TF-positive platelets that we observed in vitro (both with Meg-platelets and with $CD34^{+}$-derived platelets) was virtually identical to the amount found in blood from healthy individuals. This striking data suggests that a finely tuned mechanism, which deserves further investigation to uncover the molecular pathways involved in its regulation, is responsible for the controlled delivery of TF from megakaryocytes to platelets. All together these data support the concept that, under physiological conditions, megakaryocytes are committed to release into the bloodstream a well-defined and programmed number of TF-carrying platelets. It can be speculated that under pathological conditions, such as in the presence of low-grade inflammation as present in CAD patients, alterations in the megakaryocyte transcriptome and proteome as well as in the release of new platelets may occur. Alternatively, mechanistic events may take place in the bloodstream that drive TF pre-mRNA processing and de novo protein synthesis (►**Fig. 2**). The common consequence of these two hypothetical mechanisms is a higher number of circulating TF-positive platelets.

Although further studies are needed to prove these hypotheses in the context of CAD, we have recently observed that in spontaneously hypertensive stroke-prone rats the percentage of circulating TF-positive platelets directly correlated with blood pressure and is the result of an increased number of TF-positive megakaryocytes which release into the bloodstream a higher number of TF-positive platelets (M. Camera, PhD, unpublished data, 2015).

Although platelets do not have a nucleus, they contain about 2,000 to 7,000 transcripts.[51–53] The finding that platelets can use their mRNA pool to perform new protein synthesis in response to cellular activation is of great importance, since these mechanisms allow platelets to modify their protein phenotype and, as a consequence, their functions.[46]

Concept of Platelet Heterogeneity: 30% — The “Magic” Number

Leaving aside the controversy and giving credit to the fact that TF is really present and functional in platelets, based on the literature reviewed so far, we should also consider that TF is

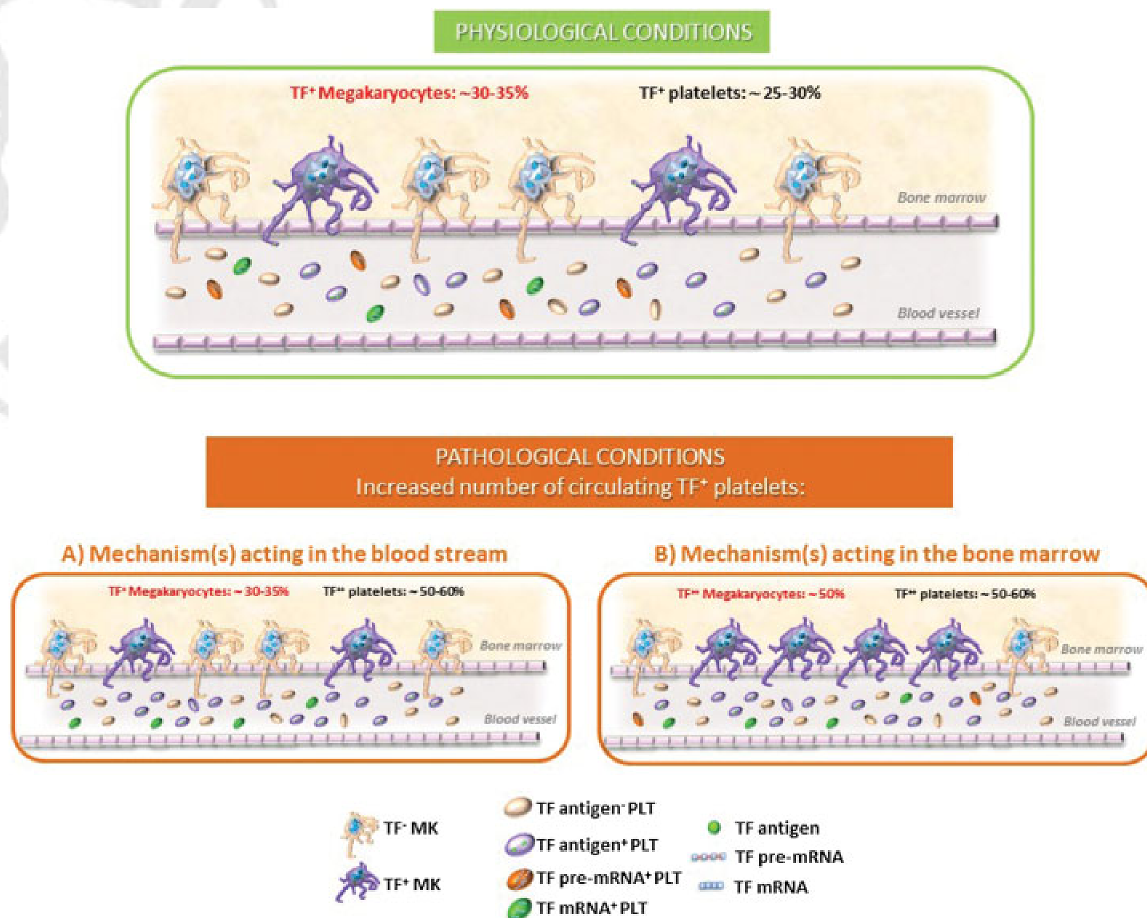

Fig. 2 Hypothetical mechanisms involved in the regulation of the circulating levels of tissue factor (TF)-positive platelets under physiological and pathological conditions. Under physiological conditions a percentage of TF expressing megakaryocytes are committed to release in the bloodstream a constant number of platelets containing either TF protein or TF pre-mRNA.[50] Under pathological conditions the increase in the number of TF-positive platelets may be the result of a (A) mechanism taking place in the bloodstream and resulting in the splicing of the TF pre-mRNA and de novo protein synthesis or (B) a mechanism taking place in the bone marrow inducing more megakaryocytes to express TF and to release TF-positive platelets.

one among the other coagulation factors, such as FV, FIX, FXI, FXIII, fibrinogen, FVII, exposed on the cell membrane upon platelet activation. It is of interest to note that for some of these factors, including TF, the presence not only of the protein, but also of the specific pre-mRNA/mRNA has been described and, as already mentioned, this is of particular relevance taking into account the biosynthetic capacity of platelets (►**Table 1**).[37,41,42,54–59]

It is well established that platelets play a key role in the initiation and regulation of hemostasis and thrombosis. Platelets adhere and aggregate at the site of vascular injury to prevent blood loss and form the building blocks of a thrombus; they also support the binding of the coagulation factors involved in tenase and prothrombinase complex (which results in thrombin generation and further platelet activation) to localize, amplify, and control the burst of thrombin generation at the injury site thus preventing a systemic activation of the blood coagulation.[60,61]

Although this function has been historically ascribed to all platelets, it is important to underline that much evidence accumulated in the past years indicate that platelets are heterogeneous in their functions and only a subset of platelets is involved in the hemostatic process.[61,62] The mechanisms of origin and function of this heterogeneity is still unclear. Indeed, as a matter of fact, individual platelets differently respond to agonists like collagen or thrombin resulting in a different binding capacity to coagulation factors and other plasma proteins. As examples in support to this concept, the platelet phospholipid asymmetry and the platelet-derived pool of FV will be briefly discussed.

Phospholipids containing choline, such as sphingomyelin and phosphatidylcholine are the main components of the outer surface of plasma membranes of all eukaryotic cells under resting conditions, while phosphatidylserine and phosphatidylethanolamine are localized on the inner surface.[63,64] In this conformation, the platelet membrane provides a nonprocoagulant surface.[65] Upon platelet activation the flip–flop mechanism, catalyzed by specific translocases,[66] allows an enhanced expression of negatively charged phosphatidylserine in the outer membrane leaflet, conferring to platelets the procoagulant surface necessary for the assembly and activation of several enzyme complexes of the coagulation process.[60,67,68] It is important to consider in this regard that, when platelet from healthy subjects are activated by physiological concentrations of agonists such as thrombin, collagen, or by the combined action of thrombin and collagen the exposure of phosphatidylserine occurs only in a fraction of activated platelets that ranges from approximately 4 to 30%, at most, depending to the agonist used.[69–71] These data clearly highlight the fact that only a subset of the activated platelets undergoes phosphatidylserine exposure on the membrane surface to sustain the hemostatic process.

Thrombin generation occurs via prothrombinase, a stoichiometric complex of the cofactor FVa and the serine protease FXa, assembled on a phosphatidylserine-exposing membrane surface in the presence of calcium.[72] FV exists in two pools in whole blood: 80% is in the plasma and 20% is stored in platelet α-granules. The α-granules stored FV is directly derived from megakaryocytes through endocytosis.[73,74] To understand the physiological role of the α-granules stored FV as compared with the plasma FV, it has been calculated that the nominal FV concentration of platelets exceeds that of plasma by an approximate factor of 100. Thus, its release from α-granules could provide a high local concentration of FV that may be critical for the generation of platelet prothrombinase activity.[75] In 2010, Fager et al published the evidence that only a fraction, about 30%, of activated platelets expressing P-selectin do also express FV, and in the same experimental conditions only a fraction of activated platelets do bind to FXa. These data support the concept that the ability of activated platelets to generate thrombin via prothrombinase is defined, once again, only by a subpopulation of platelets expressing both nondissociable and dissociable pools of platelet-derived FVa, each capable of binding FXa, and expressing an increased density of adhesive receptors, including P-selectin, at their activated membrane surface.[76]

Table 1 Coagulation factors present in platelets

Coagulation factor	Protein	mRNA	References for mRNA
Tissue factor	+	+	37,39,41,42
FV	+	Not reported	
FVII	+	Not reported	
FIX	+	Not reported	
FXI	+	+	59
FXIII	+	+	54,55, and M. Camera, PhD, unpublished data, 2015
Fibrinogen	+	not reported	
vWF	+	+	56 and M. Camera, PhD, unpublished data, 2015
TFPI	+	+	57 and M. Camera, PhD, unpublished data, 2015
Protein S	+	+	58

Abbreviation: F, factor.

Similarly to what described for phosphatidylserine and FV, also TF is expressed only by a fraction of platelets. As shown in ►Fig. 3, flow cytometry analysis of thrombin receptor activating peptide 6 (TRAP-6) or ADP-stimulated platelets from healthy subjects shows that the expression of activated GpIIb/IIIa, of P-selectin and of TF occurs in about 80, 50, and 30% of platelets, respectively. A similar trend was observed when other platelet agonists, such as collagen or thromboxane A_2, were used. These observations fit particularly well with the in vivo evidences published by Stalker et al who reported that platelet activation is not uniform throughout the hemostatic plug.[77] They found that the hemostatic response produces an organized structure, composed of activated platelets interspersed with fibrin, characterized by a regional heterogeneity due to differences in platelet activation state, packing density, and stability. Indeed, a core of fully activated platelets, expressing P-selectin and sustaining thrombin generation, is overlaid with an unstable shell of less activated, P-selectin negative, platelets. It is noteworthy, in this regard, that when costaining for TF and P-selectin is performed on in vitro agonist stimulated human platelets, flow cytometry, and confocal microscopy analysis indicate that all the TF-positive platelets also express P-selectin (►Fig. 4).[15] Moreover, the fact that in vitro data show a colocalization between TF and FV (►Fig. 4) unequivocally combines the discoveries made by different research groups in the past years. All together these data strongly support the in vivo hemostatic plug model reported by Stalker et al, according to which the thrombin generation capacity is restricted to the platelets composing the core of the plug,[77] and they also give further credit to the theory proposed by the Nemerson group 15 years ago.[6,34]

Electron microscopy analysis of thrombi formed in vivo published in the 1960s provided the first evidence for the presence of a heterogeneous platelet activation within the thrombus.[78–80] More recently, Palmerini et al assessed the presence of coagulant active TF in coronary thrombi collected from patients with ST-segment elevation acute

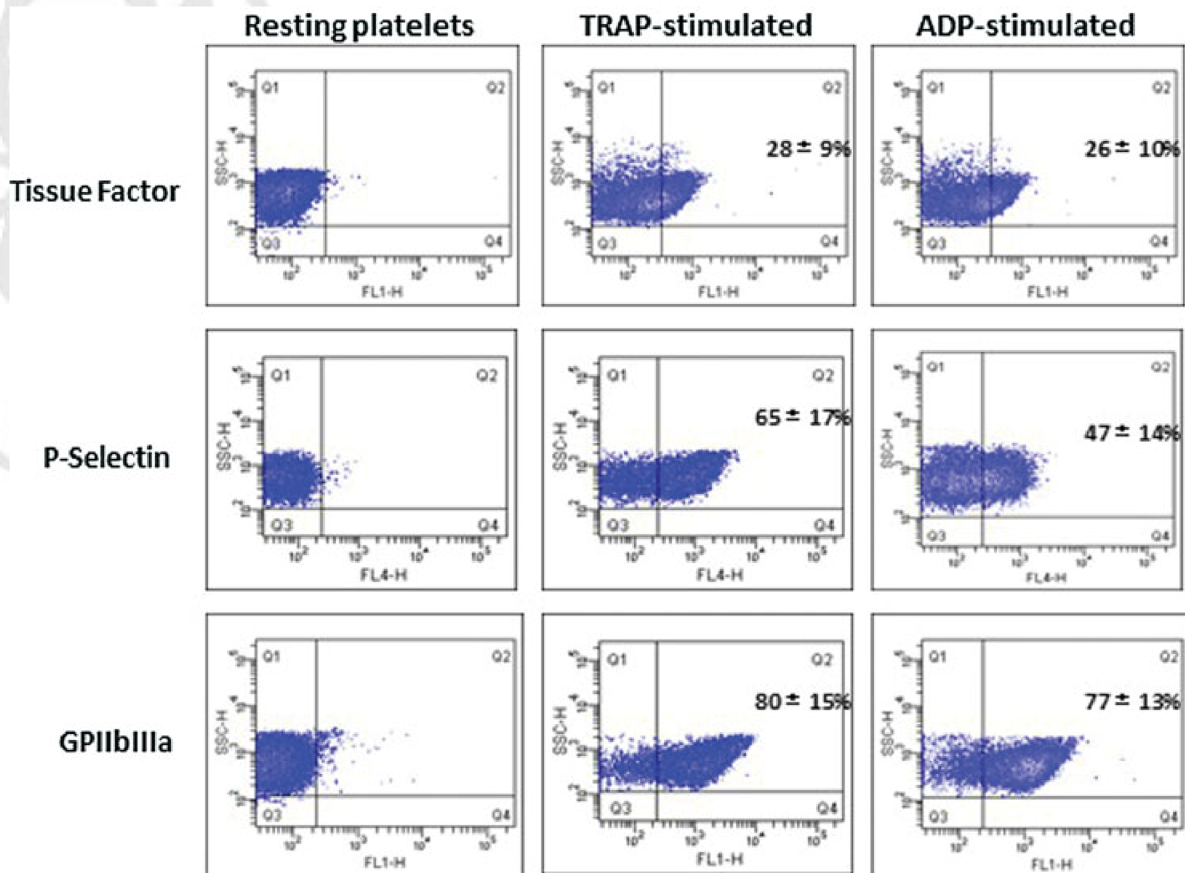

Fig. 3 Heterogeneity of platelet response to agonist stimulation. In vitro platelet activation results in a differential expression of the common markers of platelet activation, such as tissue factor, P-selectin or activated glycoprotein IIb/IIIa (GpIIb/IIIa), identifying different platelet subpopulations. While almost all stimulated platelets express activated GpIIb/IIIa upon stimulation, only a subset of platelets expresses P-selectin and tissue factor (~50 and 30%, respectively). Whole blood from healthy subjects, left untreated (resting) or stimulated with TRAP-6 (5 μM) or ADP (10 μM) for 15 minutes, was incubated with saturating concentration of mouse anti-activated GpIIb/IIIa-FITC (PAC-1 monoclonal antibody, Becton Dickinson, Sparks, MD) anti-P-selectin-APC (monoclonal antibody against CD62, Becton Dickinson) or anti-TF-FITC (monoclonal antibody against TF, catalogue no. 4508, American Diagnostica, Lexington, MA) and mouse anti-CD41-PE MoAbs. FITC-, APC-, and PE-conjugated isotype controls were used in all the experiments to quantify the background labeling. Representative density plots of three independent experiments are reported in the figure. The mean value of the results is reported as percentage positive events ± standard deviation. ADP, adenosine diphosphate; TRAP-6, thrombin receptor activating peptide 6.

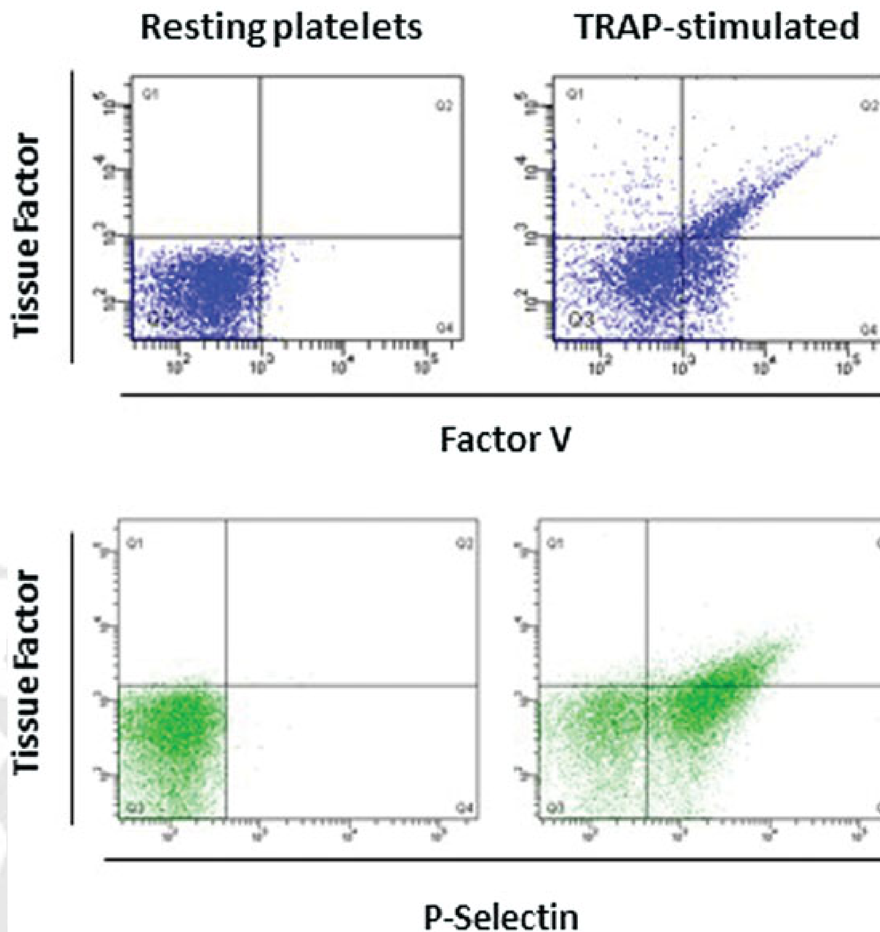

Fig. 4 Colocalization of tissue factor (TF) and coagulation factor V (FV) or P-selectin on the surface of activated platelet. Platelets expressing TF upon agonist stimulation do also express FV and P-selectin. Whole blood was stimulated in vitro with TRAP-6 (5 μM, 15 minutes) and labeled for TF and coagulation FV (Alexa Fluor 647 labeled monoclonal antibody against FV, Enzyme Research, Life Technologies, Grand Island, NY) or P-selectin. The representative density plots of three independent experiments reported in the figure show the colocalization of TF with coagulation FV (12 ± 8%) or with P-selectin (20 ± 5%). TRAP-6, thrombin receptor activating peptide 6.

myocardial infarction (STEMI), showing that functionally active TF accumulates at sites of thrombus formation in the acute phase of STEMI; the TF activity was indeed specifically inhibited by a monoclonal antibody able to block TF-mediated coagulation activity.[81] Although, as stated by the authors, the study was not designed to assess the source of TF found in thrombi, Palmerini et al showed very clearly that, in a human setting, also platelets, besides inflammatory cells within the thrombus of STEMI patients, stain positively for TF. These findings confirm leukocytes as source of TF, but they also support the evidence that platelets may be another source of TF. Of note, the TF immunolocalization provided by Palmerini et al highlights that not all platelets within the thrombus express TF. These data completely match with the evidence, as already stated above, that not all circulating platelets, both in healthy subjects as well as in patients with CAD, express TF when analyzed by flow cytometry.[37,39]

Species-Specific Differences in Platelet Tissue Factor Expression

The dispute centered on platelet-associated TF has also been fueled over time by the fact that the evaluation of the expression of platelet-associated TF in mice provided negative results. By contrast, compelling evidence supports the presence of TF in rat platelets. Indeed, in 2012 Hernández Vera et al showed, through an immunological approach, the presence of functionally active TF in rat platelets.[82] More recently, Tyagi et al not only provided the evidence, using a proteomic approach, that exposure of rats to a hypoxic stimulus results in an upregulation of platelet-associated TF expression but they also showed that platelets contain TF pre-mRNA which is processed, upon exposure to hypoxia, to form the mature TF mRNA.[82,83] Whether the negative results in mice and the positive results in rats are a matter of species specificity or a methodological issue remains to be addressed. It should be mentioned, however, that important cross-species differences in mammalian coagulation systems have been previously reported.[84,85] Moreover, although several antibodies able to recognize different epitopes of the human TF protein have been developed and are commercially available, much more limited is the availability of antibodies directed against mouse TF.[86] Finally, it is worth mentioning that TF protein was also observed in platelets from pigs. Of interest, platelets from ovariectomized pigs treated with 17β-

estradiol or with raloxifene showed increased TF levels compared with untreated pigs.[87]

Conclusions and Future Directions

Although the concept of platelet-associated TF has not been completely accepted so far in the field of hemostasis and thrombosis, the evidence of its presence and its potential involvement in the hemostatic and thrombotic processes both in humans and in experimental animal models, has accumulated in the past 15 years.[37,38,43–45] In one hand, the evidence of the present time suggests that the function of platelet-associated TF is to support thrombus growth, as first postulated by the Nemerson group, in the setting of a plaque rupture with thrombotic occlusion.[6,34] On the other hand, platelet-associated TF could play a key role also in venous thrombosis and in immune defense where activation of platelets and the coagulation cascade take place in the absence of endothelial injury and, therefore, in the absence of the contribution of the vessel wall-derived TF.[88,89] The next chapter of the evolving story of platelet-associated TF will include the identification of the molecular mechanisms involved in its overexpression in pathological conditions. Such mechanisms should allow for the development of new therapeutic strategies aimed to the prevention of thrombotic complications in cardiovascular diseases.

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