Tissue Factor Prothrombotic Activity Is Regulated by Integrin-arf6 Trafficking

Andrea S. Rothmeier, Patrizia Marchese, Florian Langer, Yuichi Kamikubo, Florence Schaffner, Joseph Cantor, Mark H. Ginsberg, Zaverio M. Ruggeri, Wolfram Ruf

**Objective**—Coagulation initiation by tissue factor (TF) is regulated by cellular inhibitors, cell surface availability of procoagulant phosphatidylserine, and thiol-disulfide exchange. How these mechanisms contribute to keeping TF in a noncoagulant state and to generating prothrombotic TF remain incompletely understood.

**Approach and Results**—Here, we study the activation of TF in primary macrophages by a combination of pharmacological, genetic, and biochemical approaches. We demonstrate that primed macrophages effectively control TF cell surface activity by receptor internalization. After cell injury, ATP signals through the purinergic receptor P2rx7 induce release of TFα microvesicles. TF cell surface availability for release onto microvesicles is regulated by the GTPase arf6 associated with integrin α4β1. Furthermore, microvesicles proteome analysis identifies activation of G×α5 as a participating factor in the release of microvesicles with prothrombotic activity in flowing blood. ATP not only prevents TF and phosphatidylserine internalization but also induces TF conversion to a conformation with high affinity for its ligand, coagulation factor VII. Although inhibition of dynamin-dependent internalization also exposes outer membrane procoagulant phosphatidylserine, the resulting TFα microvesicles distinctly lack protein disulfide isomerase and high affinity TF and fail to produce fibrin strands typical for microvesicles generated by thrombo-inflammatory P2rx7 activation.

**Conclusions**—These data show that procoagulant phospholipid exposure is not sufficient and that TF affinity maturation is required to generate prothrombotic microvesicles from a variety of cell types. These findings are significant for understanding TF-initiated thrombosis and should be considered in designing functional microvesicles-based diagnostic approaches.

**Visual Overview**—An online visual overview is available for this article. (Arterioscler Thromb Vasc Biol. 2017;37:1323-1331. DOI: 10.1161/ATVBAHA.117.309315.)

**Key Words:** dynamins ■ extracellular vesicles ■ fibrin ■ macrophages ■ proteome

Tissue factor (TF) initiates extrinsic coagulation by serving as the cell surface receptor for coagulation factor VIIa (FVIIa). In cells of the myeloid-monocytic lineage, which are central to the crosstalk of inflammation and coagulation, TF remains predominantly in a noncoagulant, encrypted form. Secondary signals can activate TF procoagulant function, and thus cause thrombotic complications in disease. Relevant injury and danger signals in this context are degradation of TF pathway inhibitor by neutrophil proteases, complement activation, protein disulfide isomerase (PDI) release by injured cells, and triggers of neutrophil extracellular traps incorporating TF.

In addition, extracellular ATP generated by tissue injury and inflammation activates the purinergic receptor P2rx7 that stimulates TF activity on smooth muscle cells, macrophages, and human dendritic cells. Primarily recognized for promoting the release of the proinflammatory cytokine interleukin-1β, P2rx7 signaling also induces cell surface exposure of phosphatidylserine and the release of highly procoagulant TF on microvesicles. Thiol-disulfide exchange reactions depending on the thioredoxin—thioredoxin reductase system are crucial for both intracellular caspase 1 activation and extracellular reductive changes inducing TF activation and microvesicles release. Thus, P2rx7 signaling is a central mechanistic link in the coupling of inflammation and coagulation.

Thiol-disulfide exchange and PDI have been implicated in regulating TF procoagulant activity. However, phosphatidylserine exposure is also influenced by PDI and concomitantly induced by stimuli that promote TF activation, thus it remains controversial whether TF structural changes induced by PDI are relevant for enhancing TF procoagulant activity or whether phosphatidylserine exposure is sufficient. Alternative conformations of TF are indicated by measurements of affinity for its ligand, FVIIa. For example, TF–FVIIa signaling requires FVIIa.
concentrations of 5 to 10 nmol/L, whereas TF-dependent FX activation can be efficient with FVIIa at pM concentrations, suggesting that the procoagulant pool of cell surface TF has higher affinity for FVIIa.13,24 TF with high affinity for FVIIa has been demonstrated in cell-binding studies on highly procoagulant monocytes and other cells.25,26 However, experimental approaches have so far not clearly dissected functional contributions of TF affinity versus procoagulant phospholipids in the context of physiologically relevant membranes.

Although the functional crosstalk of TF with integrins in cell signaling and cancer progression is well established,27,28 we report here that the arf6 recycling pathway29 that controls integrin α4β1 function in macrophages30 also regulates TF surface availability. Thus, we found that ATP stimulation of P2rx7 uncouples the TF-integrin α4β1 complex from arf6 control, enabling TF incorporation into microvesicles. Intervening at distinct points in these TF trafficking pathways, we have been able to dissect prothrombotic effects caused by procoagulant phospholipid exposure on microvesicles from those of enhanced TF affinity for FVIIa required for prothrombotic activity in flowing blood.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

Internalization Controls TF Cell Surface Activity

Cell surface TF activity in interferon γ- and lipopolysaccharide (LPS)-primed, adherent macrophages is low in the absence of secondary stimuli.13 We followed antibody-tagged cell surface TF in primed macrophages carrying knocked-in human TF (TFKI macrophages)31 and found that constitutive internalization of cell surface TF was prevented by the dynamin inhibitor Dynasore32 (Figure 1A; Figure IA in the online-only Data Supplement). Dynamin controls both clathrin- and raft-dependent internalization.33 Dynasore treatment increased cell surface phosph(211,193),(338,234)

Supplement), indicating mobilization of a similar lipid raft-localized pool of cell surface TF.

We previously showed that the release of TF+ microvesicles carrying γ-actin and PDI after P2rx7 activation occurred from filopodia.11 Dynasore treatment did not promote the formation of filopodia (Figure IC in the online-only Data Supplement), and, consistently, Dynasore-induced microvesicles lacked surface actin characteristic of ATP-induced microvesicles and carried markedly reduced levels of PDI relative to TF and integrin β1 (Figure 2A and 2B). We have previously shown that caspase-1 promotes TF trafficking and release on thrombo-inflammatory microvesicles carrying proteins with free thiol groups. We, therefore, hypothesized that microvesicles thiol-proteome analysis might uncover additional components of cellular events required for the generation of TF+ microvesicles. The macrophage cell line RAW 264.7 facilitated scale up of microvesicles protein recovery for mass spectrometry. Using this method, we identified Gαq, 14-3-3ε, and 14-3-3β/α in the indicated thiol-labeled positions as constituents of ATP-induced microvesicles (Figure IIA in the online-only Data Supplement). These proteins were of interest, since the activation of Gαq is known to promote secretion34–37 and 14-3-3ε and β/α are inhibitors of the Gαq-specific regulator of G-protein signaling 16 (RGS16).38

Western blotting furthermore showed that primary macrophages expressed only low levels of 14-3-3β/α, 14-3-3ε and the predicted partner RGS16 were detected in the microvesicles fraction of ATP-stimulated macrophage (Figure 2C), but these proteins were absent from Dynasore-induced microvesicles (Figure IIB in the online-only Data Supplement). Gαq12, but not Gαq13, was primarily detected in the microvesicles-free cell supernatant fraction from ATP-stimulated primary macrophages (Figure 2C), suggesting that TF release was associated with the activation of selected G-proteins by P2rx7 stimulation. Inhibition with pertussis toxin was not a feasible approach to demonstrate functional involvement of Gαq signaling in TF release because this treatment is known to interfere with LPS priming and TF induction.39 We, therefore, evaluated the contributions of Gαq12 activation to TF+ microvesicles release by stimulating macrophages with mastoparan, a direct activator of Gαq12.40,41 Similar to ATP stimulation, mastoparan treatment led to release of Gαq12 into the microvesicles-free extracellular space (Figure 2D), and FACS analysis showed that mastoparan generated large quantities of TF and phosphatidylserine-positive microvesicles (Figure IIC and IID in the online-only Data Supplement). Mastoparan, but not the inactive control peptide m17, induced microvesicles release of TF, integrin β1, PDI, and γ-actin as well as 14-3-3ε and RGS16 (Figure 2D). These data provide evidence that Gαq12 is a downstream component of P2rx7 signaling and suggest a possible role for Gαq12 in a push reaction for release of microvesicles, once TF and integrins associate with PDI- and actin-containing microdomains.

Integrin-associated arf6 Controls TF Availability on the Cell Surface

We next analyzed the released proteome to better understand the differences between microvesicles generated by ATP stimulation versus dynamin blockade. Coomassie brilliant
blue–stained gels showed a protein that was preferentially released into the supernatant of ATP-stimulated cells (Figure IIIA in the online-only Data Supplement). Mass spectrometry identified this protein as the small rho GTPase arf6. Western blotting confirmed that cellular levels of arf6 were markedly decreased after ATP stimulation and that arf6 was selectively released into the supernatant of ATP-stimulated cells (Figure 3A). Strikingly, arf6 was also depleted from Dynasore-stimulated cells, but predominantly recovered in the microvesicles fraction, suggesting that transport of TF and integrins was mediated by arf6 and that P2rx7 activation induced dissociation of arf6 during microvesicles release. Consistently, paxillin, an integrin adaptor protein that recruits arf6 regulators, was found at similar levels in the microvesicles fraction of ATP- and Dynasore-stimulated cells (Figure 3A).

Integrins α4β1 and α5β1 are known ligands for TF and are expressed by proinflammatory macrophages. FACS analysis showed that both integrin α subunits were equally expressed on the cell surface of quiescent macrophages, but upon ATP or Dynasore stimulation, integrin α4 levels decreased markedly, whereas integrin α5 surface levels remained unchanged (Figure 3B). Conversely, we detected integrin α4, but only low levels of integrin α5, on released microvesicles, suggesting that α4β1 traffics together with TF and represents the major integrin β1 heterodimer on TF+ microvesicles.

We, therefore, studied integrin α4SA macrophages that express an integrin α4 mutant defective in phosphorylation-induced paxillin dissociation. Paxillin recruits negative regulators of arf6, leading to the inactivation of arf6 in the receptor complex. Of note, FACS staining showed similar integrin α5 expression by wild-type (wt) and α4SA macrophages (113%±10% of wt; P=0.25, Wilcoxon signed-rank test, n=3), whereas integrin α4 levels were marginally increased in the mutant (145%±6.4% of wt; P=0.13, Wilcoxon signed-rank test, n=4). Because arf6 is implicated in macrophage filopodia formation and cancer cell microvesicles release, we investigated whether integrin α4SA macrophages differed from wt macrophages in their ability to generate microvesicles. wt and integrin α4SA macrophages showed similar ATP-induced filopodia formation with visible TF staining (Figure IIIB in the online-only Data Supplement) and generated similar amounts of microvesicles (Figure 3C), demonstrating that microvesicles release was not measurably altered in α4SA macrophages.

ATP-induced microvesicles released from wt and mutant macrophages carried similar high levels of integrin α4 and low levels of integrin α5 and showed higher TF procoagulant activity (Figure III C and IID in the online-only Data Supplement).
phosphatidylethanolamine. Blocking phosphatidylethanolamine
germinated microvesicles formed fibrin strands originating at the TF-negative macrophages (Figure 2). Remarkably, ATP-induced microvesicles preincubated with 10H10 had no effect on prothrombinase activity of ATP-induced microvesicles (109±13%, P=0.255; Wilcoxon signed-rank test, n=6), excluding indirect effects of the antibody on microvesicles membrane lipid procoagulant properties. Furthermore, thiol labeling of microvesicles was not altered by 10H10 treatment (Figure 5C), indicating that anti-TF 10H10 allosterically forces microvesicles-localized TF to adopt a low affinity conformation for ligand binding and sterically interferes with FVIIa concentrations on released microvesicles. This effect was not prevented by the PDI inhibitor PACMA31 (Figure 5B). Anti-TF 10H10 had no effect on prothrombinase activity of ATP-induced microvesicles (109±13%, P=0.255; Wilcoxon signed-rank test, n=6), excluding indirect effects of the antibody on microvesicles membrane lipid procoagulant properties. Furthermore, thiol labeling of microvesicles was not altered by 10H10 treatment (Figure 5C), indicating that anti-TF 10H10 allosterically forces microvesicles-localized TF to adopt a low affinity conformation for ligand binding and sterically interferes with FVIIa concentrations on released microvesicles.

Prothrombotic Properties of Microvesicles Carrying TF With High Affinity for FVIIa
We next evaluated whether the release mechanism of TF was important for microvesicles functional properties in flowing blood. We had previously shown that fibrin formation is induced dependent on TF after the addition of ATP microvesicles from mutant when compared with wt macrophages (Figure 3D). Conversely, lower levels of arf6 were released into the cell supernatant of ATP-stimulated α4SA versus wt macrophages. As expected, no phosphorylated integrin α4 was detectable in the α4SA microvesicles. ATP-induced microvesicles from α4SA macrophages carried more TF antigen, consistent with the increased TF activity. Thus, arf6 regulating the TF-associated integrin α4 influences the cell surface levels of TF and determines the availability of TF for incorporation into released microvesicles.

Consistent with the more stable interaction of integrin α4 with paxillin and arf6 in phosphorylation-deficient α4SA macrophages, both proteins were incorporated at increased levels into ATP-induced microvesicles from mutant when compared with wt macrophages (Figure 3D). Conversely, lower levels of arf6 were released into the cell supernatant of ATP-stimulated α4SA versus wt macrophages. As expected, no phosphorylated integrin α4 was detectable in the α4SA microvesicles. ATP-induced microvesicles from α4SA macrophages carried more TF antigen, consistent with the increased TF activity. Thus, arf6 regulating the TF-associated integrin α4 influences the cell surface levels of TF and determines the availability of TF for incorporation into released microvesicles.

Prothrombotic Properties of Microvesicles Carrying TF With High Affinity for FVIIa
We next evaluated whether the release mechanism of TF was important for microvesicles functional properties in flowing blood. We had previously shown that fibrin formation is induced dependent on TF after the addition of ATP microvesicles. \(^1\) TF/ phosphatidylserine/ microvesicles released from ATP- or Dynasore-stimulated cells were counted by FACS and equal amounts of microvesicles were added to blood perfused over TF-negative macrophages (Figure 4A). Strikingly, only ATP-generated microvesicles formed fibrin strands originating at the sites of localized particles and extending in the flow direction.

Microvesicles are known to incorporate outer leaflet phosphatidylethanolamine. \(^46\) Blocking phosphatidylethanolamine with 1 µmol/L Duramycin did not interfere with lactadherin binding to phosphatidylserine on microvesicles released by either stimulus, but nevertheless completely abolished TF-dependent FXa generation and prothrombinase activity of both ATP- and Dynasore-induced microvesicles (Figure IV A and IVB in the online-only Data Supplement). FXa generation by TF after activation by oxidation \(^16\) or thiol-modification \(^19\) is inefficiently inhibited by phosphatidylserine blockade with annexin 5. Similarly, the phosphatidylserine inhibitor lactadherin incompletely blocked FX activation on ATP-generated microvesicles independent of the concentrations of FVIIa used in the assay (Figure 4B). Unexpectedly, this experiment also indicated that ATP-generated microvesicles carried TF that was saturated for maximal FX activation at much lower concentrations of FVIIa than seen with microvesicles released after the blockade of internalization with Dynasore. In addition, FX activation on Dynasore-induced microvesicles was blocked with lactadherin, indicating that alternative procoagulant lipid composition is an unlikely cause for the enhanced prothrombotic activity of ATP-induced microvesicles.

Thus, TF incorporated into PDI-containing microvesicles exhibited high, subnanomolar affinity for its ligand FVIIa. ATP, but not Dynasore, microvesicles carried TF that furthermore supported FX activation at lower concentrations of zymogen FVII (Figure 4C), consistent with the known similar affinity of activated and zymogen FVII for TF. \(^47\) Thrombin generation assays in platelet-rich plasma also showed markedly decreased activity of Dynasore-induced when compared with ATP-induced microvesicles (Figure 4D). Because microvesicles generated by these agonists also differed in the composition of several proteins, we sought to identify a strategy that directly implicated TF in fibrin strand formation. We had previously shown in epithelial cells that anti-TF 10H10 preferentially inhibits and reacts with low affinity signaling pools of TF and only marginally interferes with cell surface FXa generation activity of TF. \(^15\) Unexpectedly, anti-TF 10H10 significantly inhibited the activity of TF at low, but not high, FVIIa concentrations on released microvesicles (Figure 5A). This effect was not prevented by the PDI inhibitor PACMA31 (Figure 5B). Anti-TF 10H10 had no effect on prothrombinase activity of ATP-induced microvesicles (109±13%, P=0.255; Wilcoxon signed-rank test, n=6), excluding indirect effects of the antibody on microvesicles membrane lipid procoagulant properties. Furthermore, thiol labeling of microvesicles was not altered by 10H10 treatment (Figure 5C), indicating that anti-TF 10H10 allosterically forces microvesicles-localized TF to adopt a low affinity conformation for ligand binding and sterically interferes with FVIIa interactions required for high affinity binding on microvesicles.

Remarkably, ATP-induced microvesicles preincubated with 10H10 produced markedly reduced fibrin strand formation in flowing blood (Figure 5D). Quantification of fibrin-positive particles showed that similar numbers of control and 10H10-preincubated microvesicles localized to the cell surface and initiated limited fibrin formation (Figure 5D). However, overall fibrin deposition was significantly decreased in the presence of 10H10. These data provided an independent line of evidence that the conformation of TF associated
with high affinity ligand binding, rather than the microvesicles phospholipid composition, was primarily responsible for efficient procoagulant fibrin strand formation under flow.

**P2X7 Receptor Stimulation of Smooth Muscle and Cancer Cells Induces the Release of Microvesicles Carrying TF With High Affinity for FVIIa**

To determine whether P2rx7-induced release of high affinity TF is unique for macrophages, we further analyzed microvesicles release and procoagulant activity from other cell types expressing P2X7 receptors and TF. We have demonstrated previously that smooth muscle cells activate and release TF in response to ATP-induced P2rx7 activation.12 As seen with macrophage-derived microvesicles, FX activation was maximal at subnanomolar concentrations of FVIIa, consistent with the presence of TF with high affinity for FVIIa (Figure 6A).

We furthermore isolated breast cancer cells from the polyoma middle T model of spontaneous breast tumor development.48 Cells isolated from P2rx7-deficient tumors lacked the P2rx7 protein based on Western blotting, but expressed levels of TF, integrin β1, actin, and PDI similar to wt controls (Figure 6B). As expected, wt but not P2rx7−/− cells responded to ATP stimulation with the release of microvesicles that, as previously seen with smooth muscle cells, carried the P2X7 receptor along with integrin β1 and TF. Importantly, FXa generation on these microvesicles was maximal with 0.2 nmol/L FVIIa (Figure 6C), demonstrating that injury signaling by the P2X7 receptor in a variety of cell types produces microvesicles carrying TF with high affinity for its ligand FVIIa.

**TF on Human Monocyte-Derived Microvesicles Has High Affinity for FVIIa**

To extent these findings to other pathophysiologically relevant injury signals activating TF, we studied TF function on blood monocyte-derived microvesicles. TF expressed by blood monocytes is known to be rendered fully procoagulant dependent on PDI in the context of activation of the complement pathway,3 and complement-dependent monocyte TF activation plays a pivotal role in venous thrombus development in mice.5 In addition, LPS stimulation of human whole blood causes a PDI-dependent appearance of microvesicles procoagulant activity.5 To confirm that TF release from human cells was also associated with affinity maturation, we isolated microvesicles from LPS-stimulated whole blood. We found that isolated microvesicles activated FX efficiently in a TF-dependent manner without the addition of FVIIa (Figure 6D); thus microvesicles TF apparently bound FVIIa with high affinity even in anticoagulated blood. We then used human FVIIa-specific

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**Figure 3.** Availability of tissue factor (TF) for incorporation into microvesicles (MV) is regulated by arf6. A, Representative Western blots of TF, integrin β1, and paxillin on MV, as well as of arf6 in MV, MV-free cell supernatant (Sup) and cells of control, ATP-, and Dynasore-stimulated macrophages. Bars present densitometric quantifications of Western blots from 3 independent experiments; *P<0.05, ***P<0.001, t test. B, fluorescence-activated cell sorter (FACS) detection of integrin α4 and α5 on the cell surface of macrophages after 30 minutes in assay buffer without (control), ATP, or Dynasore (Dyna) stimulation; **P<0.01 ANOVA (Tukey), n=3. Right, FACS detection of integrin α4 and α5 on MVs released from ATP- or Dynasore (Dyna)-stimulated macrophages, n=4. C, FACS detection of MV released from wild-type (wt) and α4SA macrophages stimulated with ATP for 30 minutes. Mean MV counts were indistinguishable between phenotypes (wt: 1611±774, α4SA: 1601±353, mean±SD, n=3). D, Western blots of TF, phospho-integrin α4, paxillin, γ-actin, and protein disulfide isomerase (PDI) on MV and arf6 on MV, MV-free cell supernatant or cells of wt and α4SA ATP-stimulated macrophages. Bars represent densitometric quantifications of Western blots from 3 independent experiments; *P<0.05, **P<0.01, ***P<0.001, t test.
monoclonal antibodies to dissociate the TF–FVIIa complex on isolated microvesicles and obtained an estimate of TF affinity for FVIIa binding by using mouse FVIIa that shows no specific activity in the presence of 0.2 nmol/L coagulation factor VII (FVIIa; closed bars) and 10 nmol/L FVIIa (open bars) in the presence of 10H10 (50 µg/mL); **P<0.05, paired t test, n=3. B, FXa generation by ATP-induced microvesicles with 0.2 nmol/L FVIIa in the presence of 10H10 (50 µg/mL) or PACMA31 (10 µmol/L); ***P<0.001, ANOVA (Tukey), n=3. C, Microvesicles from ATP-stimulated macrophages were harvested and labeled with MPB in absence or presence of 10H10 (50 µg/mL) or PACMA31 (10 µmol/L). D, Fibrin formation on TF-deficient macrophages analyzed as in Figure 4A, except that perfused blood contained microvesicles from ATP-stimulated cells preincubated for 5 minutes on ice with either control IgG1 or anti-TF monoclonal antibody 10H10 (100 µg/mL); scale bar=100 µm. For statistical calculations, projections of 3 independent experiments, each including measurements in three preselected positions in the chamber, were analyzed with ImageJ. Number of fibrin-positive particles (P=0.30, t test, n=3) (left), and total integrated density (mean gray value times surface area in pixels) of the fibrin-positive particles as measure for the amount of deposited fibrin on the surface (**P<0.05, t test, n=3) (right). n.s. indicates not significant.

Figure 5. Affinity modulation of prothrombotic tissue factor (TF) by anti-TF antibody 10H10. A, FXa generation by ATP-induced TF-negative microvesicles with 0.2 nmol/L coagulation factor VII (FVIIa; closed bars) and 10 nmol/L FVIIa (open bars) in the presence of 10H10 (50 µg/mL); **P<0.05, paired t test, n=3. B, FXa generation by ATP-induced microvesicles with 0.2 nmol/L FVIIa in the presence of 10H10 (50 µg/mL) or PACMA31 (10 µmol/L); ***P<0.001, ANOVA (Tukey), n=3. C, Microvesicles from ATP-stimulated macrophages were harvested and labeled with MPB in absence or presence of 10H10 (50 µg/mL) or PACMA31 (10 µmol/L). D, Fibrin formation on TF-deficient macrophages analyzed as in Figure 4A, except that perfused blood contained microvesicles from ATP-stimulated cells preincubated for 5 minutes on ice with either control IgG1 or anti-TF monoclonal antibody 10H10 (100 µg/mL); scale bar=100 µm. For statistical calculations, projections of 3 independent experiments, each including measurements in three preselected positions in the chamber, were analyzed with ImageJ. Number of fibrin-positive particles (P=0.30, t test, n=3) (left), and total integrated density (mean gray value times surface area in pixels) of the fibrin-positive particles as measure for the amount of deposited fibrin on the surface (**P<0.05, t test, n=3) (right). n.s. indicates not significant.

Discussion
This study provides new insights into the cellular regulation of TF procoagulant activity. We have demonstrated that primary macrophages effectively control TF cell surface activity by dynamin-mediated receptor internalization. Inhibition of dynamin not only prevents TF uptake but also promotes the release of TF-positive microvesicles. Characterization of the released soluble and microvesicles proteomes led us to identify a genetically validated pathway in which integrin α4 regulates TF cell surface availability in an arf6-dependent manner. After P2x7 activation, Go12 signaling is furthermore implicated in a push for trafficking of TF onto microvesicles with a distinct protein composition.

Arf6 has emerged as a master regulator of cell migration and adhesion by modulating intracellular trafficking of integrins. Inactivation of arf6 prevents receptor uptake and alters recycling routes, ultimately leading to the increased cell surface presence of integrins. The coordinated incorporation of TF and integrin α4β1 into microvesicles released from filopodia indicates a close association and common trafficking pathway of these receptors. Accordingly, functional inactivation of integrin α4-associated arf6 in α4β1 macrophages results in increased availability of surface TF for incorporation into microvesicles.

Cytoskeleton remodeling contributes to other microvesicles release pathways and in cancer cells, deregulated adhesive properties and increased arf6 activity seem to play a major role in generating microvesicles. Our results in untransformed macrophages indicate that arf6 rather serves to control release of TF onto microvesicles. Inactivation and cellular secretion of arf6 through injury signals seem to be required to divert integrin...
microvesicles undergoes maturation that enables binding of its ligand FVIIa with subnanomolar affinity. Previous biochemical data showed that reduction or mutational elimination of the allosteric TF Cys186–Cys209 disulfide bond reduces the affinity of TF for its ligand FVIIa. Supraphysiological concentrations of FVIIa, however, are sufficient in some, but not all cell models, to overcome the markedly diminished specific activity of reduced TF in FXa generation assays. Determination of the distance between the reduced Cys186 and Cys209 residues with molecular rulers showed a close proximity compatible not only with ligand-induced fit but also with oxidation-mediated formation of a disulfide bond. The determined redox potential of the Cys186–Cys209 allosteric disulfide furthermore predicts that thioredoxin primarily acts as a TF reductase, as experimentally shown, whereas PDI would act as an extracellular oxidase of TF. Consistently, we find that only PDI containing microvesicles carry TF with high affinity for FVIIa.

The functional importance of TF maturation to a high affinity conformation is supported by the analysis of isolated microvesicles in thrombin generation assays and when added to flowing whole blood. Microvesicles generated by Dynasore treatment carried procoagulant phosphatidylserine and phosphatidylethanolamine and had activity equivalent to ATP-generated microvesicles in a prothrombinase assay. Although Dynasore-induced microvesicles localized to cells and initiated local fibrin deposition in flowing blood, they failed to propagate fibrin strand formation typical for microvesicles generated by stimulation with ATP. Anti-TF 10H10 preferentially interacts with signaling, low affinity TF and minimally inhibits FXa generation by cell surface TF. Although anti-TF 10H10 was also noninhibitory at high FVIIa concentrations with TF on microvesicles, the antibody specifically interfered with high affinity microvesicles TF activity and markedly inhibited the prothrombotic fibrin strand formation by microvesicles in flowing blood.

The relevance of affinity maturation for TF prothrombotic microvesicles activity in blood is somewhat counterintuitive because TF activity was normal at plasma FVII concentration in the static FXa generation. Earlier studies have shown that FVII and FVIIa bind with equal, 5 nmol/L affinity to lipid-free TF captured by the 10H10 antibody. As shown by the elegant study of Sen et al, in vitro relipidation procedures used to achieve maximal TF activity not only increase affinity of TF for FVIIa but also remarkably cause a more than 2 log enhancement in the association rate of FVIIa relative to low affinity TF. The finding that TF affinity is primarily driven by the on rate rather than the off rate has implications for flow systems as opposed to static FXa generation assay with prolonged incubation times. We posit that such a rate enhancement for complex formation between FVIIa and TF becomes highly relevant for thrombogenic responses under flow not only in vitro but also under pathophysiological conditions in vivo. In addition, our data show that both ATP injury signal-induced microvesicles and complement-dependent generation of microvesicles in LPS-stimulated human whole blood carry TF with high affinity for FVIIa, indicating broad pathophysiological relevance of TF affinity maturation in thrombo-inflammatory diseases. TF+ microvesicles have emerged as potential biomarkers to identify patients
with hypercoagulable states that would benefit from thrombosis prophylaxis in various diseases. Measuring levels of circulating TF by antigen-based assays has yielded inconsistent results because these assays do not necessarily discriminate between encrypted and decrypted TF. Additional microvesicles components, for instance PDI, may be optimized for increased sensitivity toward the prothrombotic form of TF with high affinity for its protease ligand, FVIIa. Thus, the novel insights into cellular mechanisms of microvesicles generation and the heterogeneity of procoagulant TF+ microvesicles presented here provide new perspectives for the evaluation of microvesicles diagnostic strategies.

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Disclosures

None.

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Material and Methods

Cell Culture: All animal procedures were approved by the IACUC of the Scripps Research Institute. Bone marrow-derived macrophages (BMDM) were generated from total bone marrow cells of C57BL/6J, human TF knock-in (TFKI) mice¹ or Itga4 S998A (α4SA) mice² as described previously ³, and primed overnight with 100 ng/ml IFNγ (Peprotech, Rocky Hill, NJ) followed by 4 hours with 1 µg/ml LPS (S. abortus equi, Enzo Life Sciences, Farmingdale, NY). Primary smooth muscle cells were isolated from mouse lungs ⁴ and breast cancer populations were obtained by outgrowth as previously described ⁵. Tumor cells were isolated from tumor bearing wt or P2xr7-deficient mice carrying the polyoma middle T oncogene expressed from the MMTV promoter.

MP release reaction: BMDM were stimulated with 5 mM ATP (Roche Applied Science, Indianapolis, IN), 80 µM Dynasore (Sigma-Aldrich, St. Louis, MO), or 25 µM mastoparan (Sigma-Aldrich) for 30 minutes or as indicated. Cellular debris was cleared (10', 1,000 g at 4ºC) and MP collected (60', 16,000 g at 4ºC) by centrifugation for functional assays or Western-blot. Where indicated, antibodies or 25 µM PACMA31 (Tocris Bio-Techne, Minneapolis) were added during the isolation of MP. MP counts in cell-free supernatants were determined by flow cytometry. In order to achieve equal MP counts for functional assays, MP were resuspended in defined volumes based on flow cytometry quantification.

MP functional assays: TF activity was determined by adding the indicated concentrations of mouse or human recombinant FVIIa (kindly provided by L. Petersen, Novo Nordisk) and 50 nM FX (Haematologic Technologies, Essex Junction, VT) to adherent cells or to resuspended MP in HBS (10 mM Hepes, pH 7.4, 137 mM NaCl, 5.3 mM KCl, 1.5 mM CaCl₂). FXa was measured using chromogenic substrate Spectrozyme FXa (Sekisui Diagnostics, Stamford, CT). MP prothrombinase activity was measured in HBS with 10 nM FVa, 5 nM FXa and 500 nM prothrombin (Haematologic Technologies) and thrombin was quantified with chromogenic substrate Spectrozyme TH (Sekisui Diagnostics).

FACS analysis: MP in cleared supernatants were stained with 5 µg/ml anti-TF 9C3-Alexa 647 conjugate, 1 µg/ml Lactadherin-FITC (Haematlogic Technologies, Essex Junction, VT) and/or 66 nM Alexa633-conjugated phalloidin (Life Technologies) and analyzed on a LSR-II flow cytometer (BD Biosciences). Duramycin (1 µM, Sigma-Aldrich) or bovine lactadherin-FITC (50 nM, Haematologic Technologies) was added as indicated. For cell surface staining, macrophages were stained in PBS (2 mM EDTA, 1% FCS) for TF (5 µg/ml Alexa488-conjugated 21E10 ⁶⁻⁸), integrin α4 (CD49d-FITC, eBioscience, San Diego, CA), or integrin α5 (CD49e-FITC, eBioscience) followed by fixation (PBS, 2 mM EDTA, 1% FCS, 1% formaldehyde). FACS data were analyzed using the FlowJo software (Tree Star, Ashland, OR).

Microscopy: Cell surface PS exposure was quantified with FITC-labeled annexin 5 (BD Biosciences) in the provided staining buffer. Images were taken at 10x magnification, and analyzed with ImageJ (http://rsweb.nih.gov/ij). For confocal microscopy, cells were stained as described previously ³ using a cocktail of Alexa 647-conjugated anti-TF antibodies 10H10, 5G9, and 9C3 (each at 5 µg/ml) ⁶-⁸ for TFKI macrophages, or affinity-purified anti-mouse TF antibody ³ for murine TF-expressing cells. After fixation cells were counterstained with phalloidin-Alexa488/647 (Life Technologies) and 1 µg/ml Hoechst (Life Technologies). Images were taken with a 63x oil emersion objective on a Zeiss 710 LSM (Carl Zeiss, Oberkochen, Germany) and processed with Image Browser (Zeiss, Jena, Germany).
Western blotting: The following antibodies were used: polyclonal anti-mouse TF, polyclonal anti-human TF, polyclonal anti-integrin β1, anti-PDI clone BD34 (BD Biosciences, San Jose, CA), anti Ga2 (EMD Millipore, San Diego, CA), anti-RGS16 (Aviva, San Diego, CA), anti 14-3-3ε and 14-3-3 α/β (Cell Signaling Technology, Danvers, MA), and from Santa Cruz Biotechnology (Santa Cruz, CA): anti-paxillin, anti-γ-actin, and anti-arf6. Cell-supernatant, MP and cells samples were prepared for Western-blotting as described previously.

Identification of MP proteins by mass spectroscopy: MP in cell-free supernatant were concentrated with an Amicon Ultra 3,000 MW (Millipore, Billerica, MA) and collected by centrifugation. Proteins were separated by SDS-PAGE and single protein bands based on the location of thiol-labeled bands on adjacent lanes were analyzed by nano-LC-MS/MS at The Scripps Research Institute Center for Mass Spectrometry.

Flow chamber experiments: Flow chamber experiments were performed as previously described with a few modifications. TF negative macrophages coated onto glass coverslips were perfused with wild-type C57BL/6J mouse blood at an initial wall shear rate of 300 s⁻¹. Blood - collected from the inferior vena cava of anesthetized mice into citrate-phosphate-dextrose buffer (12.88 mM final citrate concentration) - was mixed with anti-fibrin β-chain mouse monoclonal IgG labeled with Alexa Fluor 546 (Invitrogen, La Jolla, CA), procoagulant MP, CaCl2 (1.15 mM final concentration) and immediately perfused through the chamber for 2 minutes, followed by Dulbecco’s modified Eagle’s Medium (Lonza, Walkersville, MD) to facilitate fibrin visualization. Stacks of confocal images at 2 μm interval through the height of fluorescent structures were collected for quantifying fibrin deposition with a Zeiss 410 LSM (Carl Zeiss) and MAX 2D projections were created using ImageJ. Background was subtracted and default signal threshold was set manually for each of the repeat experiments in the particle image analysis with ImageJ.

Thrombin generation (TG) tests with platelet-rich plasma (PRP): Procoagulant activity of MPs was determined by TG tests with PRP. PRP was prepared by centrifugation of citrate anticoagulated blood at 250 g for 10 min at 25 °C and platelets were adjusted to 180·10³/μl by dilution with homologous platelet-poor plasma. PRP was mixed with resuspended MPs in HBS (10 mM hepes, 150 mM NaCl, pH 7.4) in 96-well microtiter plates. Reactions contained 360 µM benzoyloxyxycarbonyl-glycyl-glycyl-L-arginine coupled to fluorogenic 7-amido-4-methylcoumarin (Gly-Gly-Arg-AMC; Bachem Americas, Torrance, CA) as thrombin substrate; and were started by adding 18 mM CaCl2. Fluorescence was measured continuously at 37 °C for up to 40 min in a spectrofluorometer (355/460 nm excitation/emission). The rate of fluorescence intensity increase as a function of time (dF/dt) was calculated with Turbo Delphi 2006 (Borland Software Corporation, Austin, TX) and converted to thrombin-equivalent concentration (nM) using a calibration curve. The endogenous thrombin potential (ETP) of samples (i.e., total generated thrombin activity) was determined from the area under the TG curve.

Human whole blood MV characterization: Citrate-anticoagulated whole blood from healthy volunteers was stimulated with or without 10 µg/mL lipopolysaccharide (LPS; E. coli serotype 0111:B4, Sigma Aldrich, St. Louis, MO) for 4 hours at 37°C. Cell-free plasma was obtained by double centrifugation (2 x 10 minutes at 3,000g) and MV were isolated and washed by high-speed centrifugation in a microcentrifuge. TF activity was measured in a FXa generation assay with 150 nM FX and converted to arbitrary units (AU) based on a calibration curve with dilutions of lipidated recombinant human TF (Innovin®; Siemens Healthcare, Erlangen, Germany) obtained at a saturating
concentration of 5 nM FVIIa. Recombinant mouse FVIIa was kindly provided by Dr. L.C. Petersen (Novo Nordisk)\textsuperscript{10}. In order to measure TF affinity of isolated MV with prebound human FVIIa, FVIIa was dissociated with a combination of FVIIa antibodies F5-13B12, F4-2.1b and F1-3G12 (50 µg/ml each) and mouse FVIIa was added at the indicated concentrations in the FXa generation assay.

**Statistical analysis:** Data are presented as mean ± SD, unless otherwise stated. Data were analyzed with GraphPad Prism. Parametric comparison used t-Test or ANOVA with the indicated post-tests for multiple comparison.

**References**


Supplement Material

Tissue factor prothrombotic activity is regulated by integrin-arf6 trafficking

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Supplemental Figures

Figure I. Dynasore- and mastoparan- stimulated TF+ MP release. A, FACS detection of cell surface TF on macrophages kept for the indicated times in assay buffer in absence (control, closed bars) or presence (open bars) of Dynasore; ***p<0.001, t-test, n=3. B, Effect of filipin treatment on the release of procoagulant TF+ MP from ATP- and Dynasore-stimulated cells: Western blot of TF on MP (upper panel) and determination of procoagulant TF MP activity by FXa generation assay (lower panel); *p<0.05 paired t-test, n=6. Pairing efficiency test: ATP r=0.7716, *p<0.0361, and Dynasore r=0.922, **p=0.0069. C, Macrophages treated for 20 minutes with ATP or Dynasore were stained for F-actin (phalliodin-Alexa488, green), TF (αTF-Alexa647, red) and nuclei (Hoechst, blue). Images were taken on a Zeiss LSM 710 with a 63x Plan-Apochromat NA 1.4 WD 190 mm oil emersion objective and processed using Image Browser Software; scale bar = 10 µm.
Figure II. ATP and mastoparan stimulate the release of Gαi2 and TF+ MP. A, RAW 264.7 macrophage MV thiol-proteome was labeled with MPB and detected by streptavidin blot. B, Representative Western blots of TF, integrin β1, 14-3-3ε, RGS16 and caveolin on ATP- or Dynasore-induced MV. C, FACS detection of TF- and PS-labeled MV from mastoparan-stimulated cells. D, Count of TF+ PS+ MP released from macrophages stimulated for 30 minutes with ATP, Dynasore or mastoparan; ***p<0.001 ANOVA (Tukey’s), n=6.
**Figure III. Arf6 regulation of TF.**

**A**, Coomassie detection of proteins in supernatants from control, ATP and Dynasore-treated cells. The arrowhead indicates the protein band subjected to mass spectrometry. **B**, Filopodia formation and TF localization in control and ATP-stimulated wild-type (wt) and α4SA macrophages. Cell surface TF was labeled with immuno-purified rabbit anti-mouse TF antibody and detected with anti-rabbit-Alexa488 (green). Fixed cells were counterstained for F-actin (phalloidin-Alexa633, red) and nuclei (Hoechst, blue). Inserts show magnification of filopodia. Images were taken on a Zeiss LSM 710 with a 63x Plan-Apochromat NA 1.4 WD 190 mm oil emersion objective and processed using Image Browser Software; scale bar = 10 μm. **C**, Integrin α4 and integrin α5 on ATP-induced MP released from wt (open bars) or α4SA macrophages (closed bars), determined by FACS staining. **D**, FXa generation of cells and MP from ATP-stimulated wild-type or α4SA macrophages; *p<0.05, **p<0.001, paired t-test, n=14. Pairing efficiency test: cells r=0.9251, ***p<0.0001, and MP r=0.7104, **p=0.0022.
Figure IV: Prothrombotic properties of MP. A, FACS analysis of ATP- or Dynasore-induced MP treated with PE-binding duramycin (1 µM) and stained with PS-binding Lactadherin-FITC, or IgG Alexa488. B, Prothrombinase activity (left panel) and FXa generation measured with 2 nM FVIIa (right panel) on MP from ATP- or Dynasore-induced MP in the presence of duramycin (1 µM) or lactadherin (50 nM), *p<0.05, ANOVA (Turkey’s), n=3. C, Quantification of endogenous thrombin potential (ETP) from curves as shown in Figure 4D, n = 3, ***p< 0.001 t-test.