Microparticles in Hemostasis and Thrombosis

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Abstract: Blood contains microparticles (MPs) derived from a variety of cell types, including platelets, monocytes, and endothelial cells. In addition, tumors release MPs into the circulation. MPs are formed from membrane blebs that are released from the cell surface by proteolytic cleavage of the cytoskeleton. All MPs are procoagulant because they provide a membrane surface for the assembly of components of the coagulation protease cascade. Importantly, procoagulant activity is increased by the presence of anionic phospholipids, particularly phosphatidylserine (PS), and the procoagulant protein tissue factor (TF), which is the major cellular activator of the clotting cascade. High levels of platelet-derived PS\(^+\) MPs are present in healthy individuals, whereas the number of TF\(^+\), PS\(^+\) MPs is undetectable or very low. However, levels of PS\(^+\), TF\(^+\) MPs are readily detected in a variety of diseases, and monocytes appear to be the primary cellular source. In cancer, PS\(^+\), TF\(^+\) MPs are derived from tumors and may serve as a useful biomarker to identify patients at risk for venous thrombosis. This review will summarize our current knowledge of the role of procoagulant MPs in hemostasis and thrombosis. (Circ Res. 2011;108:1284-1297.)

Key Words: microparticles ■ procoagulant ■ tissue factor ■ hemostasis ■ thrombosis

Microparticles (MPs, also called microvessicles) are present in the blood of healthy individuals and are increased in various diseases, including cardiovascular disease, diabetes, sepsis, and cancer.\(^1\)\(^-\)\(^3\) They are small membrane vesicles derived from activated and apoptotic cells. MPs range in size from 100 to 1000 nm in diameter, but are typically \(\sim 200\) nm in size. They mediate cell–cell communication by transferring a cargo of cell surface receptors, mRNAs, and microRNAs from the cell of origin to target cells.\(^4\) In addition, MPs may bind and fuse with the plasma membrane of the target cell or be engulfed by the target cell.\(^4\) Importantly, MPs have been proposed to play roles in thrombosis, inflammation, and angiogenesis.\(^1\)\(^-\)\(^3\)

Platelet-derived MPs are strongly procoagulant because they contain the anionic phospholipid PS. An early study by Chargaff and West in 1946 showed that the clotting time of recalcified normal human plasma was prolonged by the removal of MPs using high-speed centrifugation.\(^5\) In 1967,
Wolf reported that activation of platelets resulted in the generation of “platelet dust” and that these platelet-derived MPs supported thrombin generation in platelet-poor plasma.6

MPs containing both PS and the procoagulant protein TF have the highest level of procoagulant activity. TF is the primary cellular activator of the clotting cascade.7 Early studies suggested that blood did not contain significant levels of TF and that all TF was expressed by extravascular cells in healthy individuals where it formed a “hemostatic envelope” around blood vessels.8 However, in 1999, Giesen and colleagues discovered that blood of healthy individuals contained very low levels of functional TF (so-called blood-borne TF). They showed that blood-borne TF contributed to thrombus formation in ex vivo models.9 TF+ MPs were observed near the surface of platelets in the thrombus.

Despite these provocative studies, whether or not blood of healthy individuals contains significant levels of functional TF remains highly controversial. Some investigators believe that there is no functional TF in unstimulated blood of healthy individuals.10 Similarly, others have failed to detect measurable TF activity in plasma or TF associated with MPs isolated from unstimulated whole blood.11 One study found that isolated MPs from healthy controls generated thrombin in a TF-independent manner.12 However, other groups have reported very low levels of TF activity in blood in the form of MPs in healthy individuals, although these levels are close to the detection limit of the assays.13–15 Recently, it was reported that 95% of TF activity in blood of healthy individuals was present on peripheral blood mononuclear cells and only 5% was present on MPs.16 Importantly, TF+ MPs can be easily isolated from a small volume of plasma. Another important consideration is that TF+ MPs in blood may be recruited to sites of vascular injury in vivo. In fact, circulating TF was found to accumulate in thrombi formed in the saphenous vein of mice but not in hemostatic clots.17 Furthermore, elegant studies by the Furie group have shown that TF+ MPs are recruited to thrombi formed in a laser-injured mouse cremaster arteriole (see below).18

Procoagulant Properties of MPs

Role of PS

The plasma membrane of normal cells has an asymmetrical distribution of lipids in the inner and outer membranes. Anionic phospholipids, such as PS, are located almost exclusively in the inner monolayer. During the formation of MPs there is loss of membrane asymmetry with ionic phospholipids being transferred to the outer membrane of the MP.19 Importantly, the presence of PS significantly increases the procoagulant activity of MPs because it facilitates the assembly of components of the clotting cascade. This is due to an electrostatic interaction between positively charged γ-carboxyglutamic acid (GLA) domains in the clotting proteins and PS on the membrane. Clotting proteins that contain a GLA domain include Factors VII (FVII), IX, and X, and prothrombin (Figure 1). PS on the surface of MPs can be detected using flow cytometry. Activated platelets generate PS+ MPs, although one study found both PS+ and PS− MPs.20

Role of TF

TF is a receptor for FVII/VIIa (Figure 1). The TF:FVIIa complex activates both FX and FIX to initiate blood coagulation.7 The presence of TF on MPs dramatically increases their procoagulant activity. TF has a high affinity for FVIIa, and therefore TF+ MPs in blood will readily bind FVII/FVIIa. The TF:FVIIa complex is regulated by tissue factor pathway inhibitor (TFPI).21 This Kunitz-type inhibitor is primarily synthesized by endothelial cells and circulates in blood to prevent inappropriate activation of the coagulation cascade.21 It inhibits the TF:FVIIa complex in a FXa-dependent manner. Therefore, it is likely that some of the TF:FVIIa complexes present on MPs in blood will be inhibited by TFPI.

Posttranslational modifications of TF, such as glycosylation, may also affect TF activity.22–24 This means that TF+ MPs from different cellular sources may have different procoagulant activities. In addition, the TF:FVIIa complex...
can be found in low- (also called encrypted) and high-activity states, which is thought to be due to differences in the conformation of TF, reviewed previously.\textsuperscript{25} The different states were discovered because disruption of TF\textsuperscript{+} cells increased TF activity without a change in TF antigen.\textsuperscript{26} This increase in TF activity was associated with an increase in PS, which led some investigators to propose that PS may induce a conformational change in TF that increases its specific activity.\textsuperscript{25} Other mechanisms of TF activation have been proposed, and it is possible that there are different mechanisms that regulate TF:FVIIa activity on MPs derived from different cell types.\textsuperscript{27}

Some investigators have proposed that MPs in blood contain TF in a low-activity state to prevent inadvertent activation of the coagulation cascade. By analogy with cells, this could be due to the fact that levels of PS on the MPs are below the optimal level for full TF activity. If this notion is correct, freezing the MPs would increase PS levels on the outer membrane and also increase TF activity. However, TF activity of MPs isolated from plasma of patients undergoing total knee arthroplasty was not increased by ionomycin treatment or freezing to increase PS exposure.\textsuperscript{16} Another group also found no difference in TF activity of fresh MPs versus frozen MPs isolated from healthy individuals and cancer patients.\textsuperscript{15} In addition, freezing MPs did not increase the level of PS (F. Dignat-George, personal communication, 2010). We prepared monocyte-derived MPs from human whole blood stimulated with bacterial lipopolysaccharide (LPS) as a model system. Consistent with other studies, frozen MPs had the same TF activity as did fresh MPs (R. Lee and N. Mackman, unpublished data, 2011). These results indicated that TF is fully active on washed MPs and that PS levels are not limiting.

### Measurement of the Levels of MPs Using Functional Assays

There are many ways to measure MPs. Flow cytometry can be used to determine the cellular origin of the different MPs, although there are concerns about the detection limit of this approach.\textsuperscript{28} Electron microscopy, atomic force microscopy, and dynamic light scattering can all be used to determine the size of MPs but do not provide information on the biological properties of the MPs.\textsuperscript{29} Measurement of TF antigen levels on MPs have been recently reviewed.\textsuperscript{30,31} We will focus on functional assays that measure the procoagulant activity of isolated MPs. Advantages of functional assays include their high sensitivity, simplicity, and the use of well-defined reagents. For instance, we found that pancreatic cancer patients had higher levels of MP TF activity than did healthy controls, whereas a TF antigen assay failed to detect a difference.\textsuperscript{14} However, functional assays do not provide any information on the cellular source of the MPs or their physical properties. Ideally, a combination of methods should be used to characterize MPs.

### PS-Dependent MP Assays

As mentioned above, the presence of PS on the surface of MPs allows assembly of the different coagulation protease complexes on the MPs. One commercial assay, called the Zymuphen MP Activity assay (Hyphen BioMed), quantifies the level of PS in the MP population.\textsuperscript{32} Briefly, PS\textsuperscript{+} MPs are captured on an ELISA plate coated with annexin V-streptavidin and incubated with FV, FX, and prothrombin to form the prothrombinase complex that cleaves prothrombin to thrombin. A chromogenic substrate for thrombin is added to assess the levels of thrombin. The values are expressed as PS equivalents. Another assay, called procoagulant phospholipid (Proag PPL) (Stago), measures the procoagulant activity of MPs added to phospholipid-free porcine plasma.\textsuperscript{33} Equal volumes of test plasma and phospholipid-free plasma are mixed before the addition of FXa, and the clotting time is measured. The level of phospholipid in the sample in the form of MPs is calculated using a standard curve prepared with synthetic phospholipids.

### TF-Dependent MP Assays

Two strategies have been used to measure TF activity of MPs isolated from plasma or capture and centrifugation. Aras and colleagues used a monoclonal antibody (1B10) to capture MPs from a variety of cell types, including monocytes.\textsuperscript{11} The level of TF activity of captured MPs is measured by adding FVIIa and FX in the presence or absence of an anti-TF antibody. Another study captured TF\textsuperscript{+} MPs using a biotinylated anti-TF antibody and then measured their TF activity by adding FVIIa and FX.\textsuperscript{34} A commercial assay called the Zymuphen MP TF assay (Hyphen BioMed) is available and captures PS\textsuperscript{+} MPs in the same way as does the Zymuphen MP activity assay. However, there are no data in the literature using this assay.

Tesselaar and colleagues used centrifugation to isolate MPs from healthy individuals and cancer patients and measure their TF activity.\textsuperscript{15} MPs are incubated with FVII, FX, and a chromogenic substrate for FXa for 90 minutes. Synthetic phospholipids are also added to the assay to provide an excess of phospholipid. Importantly, assays are performed in the presence or absence of an anti-human TF antibody to distinguish TF-dependent and TF-independent FXa generation. Levels of MP TF activity in healthy individuals was very low (132 fM Xa min\textsuperscript{−1}). In a subsequent paper, a lower level of MP TF activity was reported (4.1 fM Xa min\textsuperscript{−1}) because some of the MPs were removed by an additional centrifugation step.\textsuperscript{35} In parallel to the Osanto group, we developed a functional MP TF activity assay.\textsuperscript{14} MPs were pelleted from platelet poor plasma by centrifugation at 20,200 g for 15 minutes and washed 2 times before incubating with FVIIa and FX for 2 hours. Finally, a chromogenic substrate for FXa was added for 15 minutes. We also performed the assay in the presence of either a control antibody or an anti-TF antibody to measure TF-dependent and TF-independent FXa activity. We found low levels of MP TF activity (0.21 pg/mL) in healthy individuals. We also found that plasma preparation significantly affected the levels of MP TF activity. MP TF activity of platelet-free plasma was 64% lower than the activity of MPs prepared from platelet-poor plasma (R. Lee and N. Mackman, unpublished data, 2011). An important difference in the 2 MP TF activity assays is that we do not add exogenous phospholipids. In fact, we found that addition of exogenous synthetic phospholipids increases total FXa generation in a
TF-dependent manner (D. Manly and N. Mackman, unpublished data, 2010). These results indicate that preanalytical variables, such as plasma preparation, have a major impact on the level of TF activity of MPs. In addition, the specific activity of the recombinant TF used to prepare standard curves can vary significantly, making it difficult to compare levels of MP TF activity in different studies.

**Cellular Sources of Procoagulant MPs in Blood**

**PS⁺ MPs**

The cellular source of MPs is defined primarily on the basis of their cell surface antigens. CD41 is used to identify MP derived from platelets (Figure 2). Several studies indicate that platelets are the major source of PS⁺ MPs in blood and represent 70% to 90% of all circulating MPs.⁸,¹⁰,¹⁵,¹⁷,¹³ As noted above, activated platelets release PS⁺ MPs.⁷ The conclusion that CD4¹⁺ MPs are all derived from platelets was recently challenged because cultured megakaryocytes were found to also shed CD4¹⁺ MPs.³⁹ In fact, one study analyzed the origin of CD4¹⁺ MPs in blood using markers specific for platelets or megakaryocytes and concluded that the majority of these MPs are derived from megakaryocytes.³⁹ At present the source of the increased numbers of CD4¹⁺ MPs in different diseases is not known.

**TF⁺ MPs**

**Monocytes**

One study concluded that unstimulated monocytes do not express TF.¹⁰ However, another study found TF expression in a subset of unstimulated leukocytes and monocytes from healthy individuals.²³ Furthermore, LPS stimulation of monocytes increases TF expression and the release of TF⁺ MPs.⁸,¹⁰,⁴⁰,⁴¹ One study found that MPs from monocytic THP-1 cells expressed CD15 that mediated binding to P-selectin on activated platelets.⁴² Another study reported that THP-1 cell-derived MPs were enriched in TF and P-selectin glycoprotein 1 (PSGL-1) (Figure 2), which would allow docking onto activated platelets and endothelial cells by binding to P-selectin.⁴³ Interestingly, a recent study found that MPs derived from LPS-stimulated monocytes expressed low levels of TFPI.⁴⁴ Importantly, plasma from patients with meningococcal sepsis contained MPs that expressed TF and the monocyte marker CD14 (Figure 2).⁴⁵ More recently, Aras and colleagues reported a transient increase in MPs that express both TF⁺ and CD1⁴⁺ in a human endotoxemia model.¹³ Monocyte-derived TF⁺ MPs are also elevated in sickle cell disease.⁶⁶ These studies indicate that monocytes are likely to be the major source of TF⁺ MPs in health and disease.

**Neutrophils**

Neutrophils have also been reported to express TF in response to complement C5a.⁴⁷–⁴⁹ However, a recent study found that monocyte-derived TF⁺ MPs can readily bind to neutrophils, which may explain some of the reports of neutrophil TF expression.⁵⁰ We found that deletion of the TF gene in myeloid cells reduced fetal loss in a mouse model of antiphospholipid antibody syndrome,⁴⁹ but it was not possible to distinguish between a role for TF expression by neutrophils versus monocytes.

**Endothelial Cells**

Cultured endothelial cells express TF in response to a variety of agonists, including cytokines and LPS.⁵¹ However, there is limited evidence that endothelial cells express TF in vivo. Studies with animal models of endotoxemia and sepsis have reported TF expression in endothelial cells in the splenic vasculature and at branch points in the aorta.⁵²,⁵³ As stated above, it is possible that part or all of this TF staining is due to the binding of monocyte-derived TF⁺ MPs that are known to be present in septic animals. Indeed, TF staining of endothelial cells was restricted to granular structures that also contained the leukocyte marker PSGL-1.⁵³ Moreover, we found that a selective deletion of the TF gene in endothelial cells did not reduce the activation of coagulation in a mouse endotoxemia model.⁵⁶ However, in sickle cell mice, TF expression was observed on endothelial cells of the pulmonary veins.⁵⁵ In addition, endothelial cell–derived MPs were observed in sickle cell patients in crisis and expressed both TF and CD144 (Figure 2).⁴⁶ Interestingly, the TF activity of MPs derived from activated endothelial cells was markedly increased by inhibition of TFPI, whereas there was only a modest change using MPs from stimulated monocytes, suggesting that TF⁺ MPs from different cellular sources have different TF activity.⁵⁶,⁵⁷ These studies indicate that endothelial cells may release TF⁺ MPs in certain diseases.

**Platelets**

Platelets have been reported to express TF.⁵⁸–⁶¹ However, other investigators failed to detect any TF in resting or activated platelets.⁸,¹⁰,⁶²,⁶³ There are several explanations for these conflicting results. Some of the studies did not use inhibitory anti-TF antibodies to demonstrate that the procoagulant activity of the platelets is indeed due to TF. This is important because high concentrations of FVIIa can activate FX in a TF-independent manner.⁶⁴ In addition, the presence of TF on platelets may be due, in part, to the binding of monocyte-derived TF⁺ MPs to activated platelets. In the study by Zillman and colleagues, treatment of whole blood with collagen increased platelet TF expression.⁶¹ However,
this study did not exclude the possibility that collagen activation of the platelets exposed P-selectin on the cell surface and allowed binding of monocyte-derived TF\(^+\) MPs in the whole blood. It is more difficult to explain the reports of TF premRNA and mRNA expression in platelets and de novo synthesis of TF protein by platelets, although monocyte contamination of the platelet preparations is always a concern.\(^{59,60}\) In conclusion, platelets may express very low levels of TF, but it seems unlikely that they provide a major contribution to the pool of TF\(^+\) MPs present in healthy individuals and patients.

### Clearance of MPs

MPs have a relatively short half-life in the circulation. One study examined the role of the PS binding protein lactadherin in the clearance of platelet-derived PS\(^+\) MPs.\(^{65}\) They found that the number of CD42\(^+\) MPs was significantly higher in lactadherin-deficient mice than in wild-type (WT) littermates. In addition, splenectomized WT mice had more circulating MPs than in wild-type (WT) littermates. Another study found elevated levels of PS\(^+\) MPs in lactadherin-deficient mice.\(^{66}\) Clearance of tumor-derived human TF\(^+\) MPs was also examined in control and splenectomized mice.\(^{67}\) In control mice, peak levels of TF\(^+\) MPs were observed at 30 minutes, and none were detected at 120 minutes, whereas in the splenectomized mice, significant levels of TF\(^+\) MPs were observed at 120 minutes. Furthermore, human TF antigen was detected in the spleen. These results support the conclusion that the spleen is the major site for the clearance of PS\(^+\) MPs with or without TF.

### Role of MPs in Hemostasis

**PS\(^+\) MPs**

Platelets mediate primary hemostasis. One of the key events in platelet activation is the exposure of PS on the cell surface. By analogy, PS\(^+\) MPs derived from platelets can be viewed as a smaller version of activated platelet and express receptors for both collagen and von Willebrand factor (Figure 3). Therefore, it has been proposed that these MPs may play a role in hemostasis (Figure 3).\(^{68}\) However, it is very difficult to separate the roles of platelets and platelet-derived MPs in hemostasis because one cannot selectively remove the MPs. This is also true for megakaryocyte-derived MPs.\(^{39}\) Patients with Castaman’s defect and Scott syndrome have a bleeding tendency that appears to be due to a defect in the ability of activated platelets to translocate PS to the surface of the cells.\(^{69,70}\) Platelets from these patients also have a defect in the generation of PS\(^+\) MPs in vitro, which has been used by some to argue that platelet MPs are important for hemostasis. However, further studies are needed before it can be concluded that platelet-derived MPs are required for hemostasis.

**TF\(^+\) MPs**

After vessel injury, extravascular TF comes into contact with blood, and the clotting cascade is activated to form a hemostatic plug. Mice lacking either TF or FVII do not survive, indicating that the TF:FVIIa complex is essential for hemostasis.\(^{71}\) The clotting cascade can be divided into 2 phases: initiation and propagation.\(^{72}\) This is analogous to a stick of dynamite in which the fuse represents the initiation phase and the dynamite represents the propagation phase. The TF:FVIIa complex of the extrinsic pathway is the major trigger of the clotting cascade and generates small amounts of thrombin. In contrast, the intrinsic pathway (FXI, IX, VIIIa) is required for the burst of thrombin generation. These 2 phases can be easily separated in vitro, but clotting in vivo is more complex because of fluid flow.

So how do we fit TF\(^+\) MPs into this scheme of the clotting system and hemostasis? The low level of TF expression in a subset of unstimulated monocytes together with circulating TF\(^+\) MP may play a role in hemostasis by maintaining the idling of the clotting cascade (a low level activation that is dependent on the TF:FVIIa complex).\(^{73}\) In addition, TF\(^+\) MPs may play a role in clot growth. It has been proposed that after injury, vessel wall TF will trigger clotting but will then be covered by platelets and the clot, thus blocking it from further participation in clotting.\(^{74}\) Circulating TF\(^+\) MP may provide an alternative source of TF that would be recruited to the growing thrombus and reinitiate clotting and thus contribute to its growth.

There are arguments for and against this notion. Those investigators against the idea argue that levels of TF\(^+\) MPs in healthy individuals are too low to contribute to thrombin generation in the presence of an intact intrinsic pathway. Support for this view was provided by in vitro experiments using whole blood showing that resupply of TF to an ongoing TF-initiated clotting reaction did not enhance thrombin generation.\(^{75}\) Similarly, we found that the presence of TF\(^+\) MPs in plasma shortened the lag time but did not change the total thrombin generation in a calibrated automated thromogram assay, consistent with a role in initiation but not propagation.\(^{76}\) Those in favor of the idea that TF\(^+\) MPs contribute to clot growth argue that flow is required to deliver the TF\(^+\) MPs to the clot. One study showed that increasing the shear rate of the blood to 650 s\(^{-1}\) produced the maximal amount of TF-dependent FXa generation in ex vivo thrombi.\(^{77}\) Furthermore, leukocyte-derived TF\(^+\) MPs
have been shown to contribute to thrombus growth in an injured mouse cremaster arteriole (see below).78,79 One study found that increasing the number of circulating MPs, some of which expressed TF, restored hemostasis in a mouse model of hemophilia A.80

An added complexity to analyzing the role of TF+ MPs in hemostasis is that larger vessels contain more TF in the vessel wall than do smaller vessels. In larger vessels it has been estimated that there is a ratio in vessel wall TF to circulating TF+ MPs is 1000:1 in healthy individuals and mice.10,81 This suggests that circulating TF+ MPs are more likely to contribute to hemostasis in small vessels than in larger vessels, and also in organs that express low levels of TF, such as the liver and skeletal muscle.71 However, at present it is unclear whether the low levels of circulating TF+ MPs in healthy individuals are required for hemostasis (Figure 3). A recent study proposed that TF+ MPs present in saliva may contribute to hemostasis of superficial wounds.82

Role of Platelet-Derived MPs in Thrombosis
There are few studies that have investigated the role of platelet-derived MPs in thrombosis, although these are elevated in a number of diseases. Heparin therapy can cause heparin-induced thrombocytopenia (HIT) that is associated with decreased platelet counts but paradoxically with thrombosis. One study found that incubation of platelets with heparin and immunoglobulin induced the generation of MPs, which led to the notion that these MPs may trigger thrombosis.83 However, a more recent study showed a role for monocytes in thrombosis in HIT.84 Moreover, we found that a heparin-PF4 antibody complex induced monocyte TF expression and the release of TF+ MPs.85 These data suggest that monocyte-derived TF+ MPs rather than platelet-derived MPs may initiate thrombosis in HIT.

TF+ MPs and Activation of Coagulation in Mice and in Animal Models of Thrombosis
Previous studies have shown that the vessel wall is the major source of TF that contributes to thrombosis in a mouse model of carotid artery injury.81,86,87 However, it should be noted that these experiments were performed on healthy mice that have very low levels of circulating TF+ MPs. In fact, Reinhardt and colleagues found that mice injected with human monocyte-derived TF+ MPs had increased fibrin accumulation in a carotid artery ligation model.88 This result indicates that TF+ MPs can contribute to thrombosis in this model. However, larger numbers of MPs were used that likely far exceed the levels of circulating MPs observed in disease states. In the following sections, we will focus on studies that analyze TF+ MPs in models of activation of coagulation and thrombosis.

Role of Hematopoietic Cell-Derived TF+ MPs in a Mouse Model of Microvascular Thrombosis

Figure 4. Role of TF+ MPs in microvascular and venous thrombosis. A, Microvascular thrombosis in healthy mice. Leukocyte-derived TF+ MPs and vessel wall TF both appear to contribute to thrombosis induced by laser injury to cremaster arterioles. B, Cancer-associated venous thrombosis. Tumors release TF+ MPs into the circulation, where they are proposed to bind to activated endothelium and trigger venous thrombosis.

Induced by Laser Injury
The laser injury model of arteriole thrombosis is the best system to examine the role of MPs in thrombus formation because there is minimal injury to the vessel wall.89 This model utilizes a focused laser beam to damage the endothelium and induce thrombosis. It utilizes the microcirculation of living mice and analyzes thrombus formation by confocal microscopy. Arterioles of the cremaster muscle are typically used in this model.

Falati and colleagues observed the rapid accumulation of TF and fibrin upstream of the thrombus, consistent with recruitment of circulating TF+ MPs to the thrombus.90 However, the highest concentrations of TF were found at the thrombus–vessel wall interface, suggesting that vessel wall TF also contributed to thrombus formation. In a subsequent study, they demonstrated accumulation of ex vivo generated TF+ MPs in the developing thrombus.18 Next, they used PSGL-1 and P-selectin null mice to demonstrate that the recruitment of MP was largely dependent on the interaction of MP PSGL-1 with platelet P-selectin (Figure 4). Gross and colleagues examined the kinetics of TF incorporation into the thrombus.79 Accumulation of TF+ MPs occurred rapidly in a developing thrombus and peaked 60 seconds after the initiation of injury. By comparison, the appearance of TF+...
leukocytes first appeared 3 minutes after the initiation of injury, which demonstrates that MPs accumulate before monocytes.

To examine the cellular source of TF that contributed to thrombosis in the laser injury model, we utilized low-TF mice that only express 1% levels of human TF in comparison with WT control mice.78 Low-TF mice were found to develop small platelet-rich thrombi with reduced levels of both TF and fibrin in the laser injury model. We performed a reciprocal bone marrow transplantation to determine the role of hematopoietic cell-derived TF+ MPs in thrombus formation.78 Low-TF mice containing bone marrow from WT donors had larger thrombi with increased levels of fibrin in comparison with thrombi of low-TF mice. Conversely, WT mice containing low-TF bone marrow had smaller thrombi with less fibrin in comparison with thrombi in WT mice. These data indicated that both hematopoietic cell-derived TF+ MPs and vessel wall TF contributed to thrombus formation in this model (Figure 4). Experiments are currently being performed using mice with a deficiency of TF in myeloid cells, endothelial cells, or smooth muscle cells (SMCs) to more precisely determine the cellular sources of TF that contribute to thrombus formation in this model. Clearly, the interaction between PSGL-1 and P-selectin likely plays a key role in the recruitment of monocyte-derived TF+ MPs to the site of thrombosis in different diseases.

TF+ MPs and Animal Models of Venous Thrombosis

The role of leukocytes and TF+ MPs in venous thrombosis has been analyzed in several animal models. For example, Himber and colleagues used a model in which a collagen-coated thread is inserted in the jugular vein of rabbits and found TF-dependent fibrin accumulation and thrombus propagation.91 TF+ leukocytes accumulated in the thrombus, and it was assumed that TF+ MPs also contributed to fibrin formation in this model.

The most commonly utilized model of venous thrombosis is the inferior vena cava (IVC) ligation model (reviewed previously).92 This model is not the best model for studying the role of circulating TF+ MPs in thrombosis because delivery of MPs to the site of injury is impeded by the ligation and there is injury to the vessel wall. Ramacciotti and colleagues examined MPs in a mouse IVC ligation model.93 They found that thrombus weight correlated negatively with leukocyte-derived MPs, suggesting that these MPs were consumed by the thrombus. Moreover, injection of MPs into mice after IVC ligation increased the thrombus weight at the earlier time points. Zhou and colleagues used a rat model of IVC ligation and found a rapid accumulation of TF+ leukocytes within the thrombus in conjunction with P-selectin expression in endothelial granules.94 They further demonstrated focal areas of denuded endothelium. Biro and colleagues demonstrated that MPs, obtained from human pericardial blood following cardiac surgery, increased thrombus formation in a rat model of IVC ligation in a TF-dependent manner.95 Similar to the laser injury model, mice deficient in either PSGL-1 or P-selectin have smaller thrombi in the IVC ligation model.96 We found that low-TF mice had smaller thrombi than did control mice in the IVC ligation model.81 However, chimeric bone marrow transplantation demonstrated that hematopoietic cell-derived TF did not play a role in thrombosis. This result indicates that there are roles of PSGL-1 and P-selectin beyond mediating the docking of TF+ MPs to the growing thrombus.

Other models have been developed that are more suitable for the analysis of TF+ MPs in venous thrombosis because they maintain blood flow across the injury site that would permit delivery of circulating MPs to the thrombus.97,98 The role of hematopoietic cell-derived TF+ MPs in these models is being evaluated.

TF+ MPs and Activation of Coagulation in a Mouse Model of Endotoxemia

Recently, our group demonstrated that MP TF activity was increased in a mouse model of endotoxemia.99 Furthermore, we observed a linear correlation between MP TF activity and levels of thrombin–antithrombin (TAT) complex, a marker of the activation of coagulation. These results suggest that TF+ MPs may contribute to the activation of coagulation in this model. However, further studies are needed to ascertain the different cellular sources of TF+ MPs and their role in the activation of coagulation.

Role of TF+ MPs in the Activation of Coagulation in Tumor-Bearing Mice and Venous Thromboembolism in Cancer Patients

Venous thromboembolism (VTE) is a term used to describe both deep-vein thrombosis and pulmonary embolism. Venous thrombi occur as a result of changes in blood flow, activation of the endothelium, and changes in the blood itself. This is known as Virchow’s triad.100 The association between cancer and thrombosis has been known since the mid-19th century. Malignant tumors were found to release procoagulant plasma membrane vesicles (referred to as MPs) both in vitro and in vivo.101 Subsequent studies showed that the procoagulant activity of the MPs derived from tumor cells was due to TF.67,102,103 Importantly, circulating tumor cell–derived TF+ MPs may trigger the formation of venous thrombi in the absence of vessel injury (Figure 4).

Tumor-Derived TF+ MPs and Activation of Coagulation in Mice

Mouse studies have been used to analyze tumor-derived TF+ MPs and the activation of coagulation. For example, Yu and colleagues found that human TF antigen was released into the blood from human colorectal tumors or epithelial carcinoma cells grown subcutaneously in mice.104,105 Furthermore, the level of circulating TF was proportional to the size of the tumor, and tumors expressing higher levels of TF resulted in higher levels of circulating TF.106 Davila and colleagues also detected an increase in circulating tumor-derived TF+ MP procoagulant activity proportional to tumor size in an orthotopic pancreatic cancer mouse model.107 Recently, we found that the elevated levels of TAT in mice with pancreatic tumors were reduced by inhibition of TF with a monoclonal antimouse TF antibody (J-G Wang and N.
Mackman, unpublished data, 2011). Thomas and colleagues utilized a model of ferric chloride–induced injury of mesenteric arterioles and demonstrated a decreased time to occlusion in tumor-bearing mice and in WT mice receiving tumor cell–derived MPs.106 They also found that pancreatic tumors expressing enhanced green fluorescent protein released labeled MPs that became incorporated into thrombi. Interestingly, the tumor cell–derived MPs expressed PSGL-1 and bound to the thrombus in a P-selectin dependent manner. However, this study did not establish a role for tumor cell–derived TF+ MPs in thrombus formation. These results indicate that tumors produce procoagulant TF+ MPs, which enter the bloodstream via the leaky vasculature of the tumor and likely trigger venous thrombosis (Figure 4). Further experiments with different tumor lines are needed to analyze how tumor-derived TF+ MPs initiate thrombosis in mouse tumor models.

Clinical Studies on TF+ MPs and Cancer Patients

We will focus on studies that measured levels of TF+ MPs using flow cytometry, impedance, or activity assays, because measurement of plasma TF antigen and plasma TF activity is associated with technical problems.30,107 Recent clinical studies have demonstrated an increase in TF+ MPs in cancer patients (Table). For example, Hron and colleagues showed that patients with advanced colorectal cancer had a 2-fold increase in circulating TF+ MPs in comparison with healthy controls.38 Zwicker and colleagues found an association between TF+ MPs and cancer-associated VTE.108 Another study reported that cancer patients with VTE had higher levels of TF+ MPs in comparison with cancer patients without VTE, both of which were elevated over healthy controls.109 Finally, a patient with severe Trousseau’s syndrome had extremely high levels of TF antigen in the plasma associated primarily with MPs.110 Together, these results indicate that increased levels of MP TF antigen can be predictive of VTE in cancer patients.

Similar to the TF antigen studies, several groups have demonstrated an increase in MP TF activity in cancer patients with VTE in retrospective studies (Table). For example, Tesselaar and colleagues found increased levels of MP TF activity in patients with disseminated breast and pancreatic cancer than in healthy controls.115 In a subsequent study, they found higher levels of MP TF activity in unselected cancer patients presenting with acute VTE than in patients without VTE.111 Surprisingly, the majority of cancer patients presenting with acute VTE during chemotherapy had low levels of MP TF activity, suggesting that chemotherapy did not increase circulating MP TF activity. However, it may be necessary to collect multiple blood samples after chemotherapy to see if there is a transient increase in MP TF activity. Another study reported that patients with early-stage prostate cancer had significantly higher levels of MP TF activity than did healthy controls.112 Recently, we observed that cancer patients with VTE had significantly higher MP TF activity than did cancer patients without VTE.113 Similarly, we demonstrated that MP TF activity was elevated in patients with pancreatic or biliary cancers and this significantly correlated with VTE.114

We analyzed MP TF activity and plasma TF antigen levels prospectively in 11 advanced or metastatic pancreatic cancer patients who had repeat blood draws over a 20-week period.114 Nine of 11 patients had no significant change in MP TF activity over time but had elevated levels of MP TF activity in comparison with controls. However, 1 patient started with a low level of MP TF activity that increased in each of the subsequent blood draws and was at the highest level prior to a VTE event. Another patient started with an elevated level of MP TF activity and increased over time before a VTE. We also observed similar changes in TF antigen levels in the plasma in the 2 patients using an in-house ELISA assay. Similarly, Zwicker and colleagues found a 7-fold increase in TF antigen levels in the plasma in the 2 patients with severe VTE.115

Several studies have demonstrated an association between levels of TF+ MPs and mortality. Tesselaar and colleagues reported that high levels of MP TF activity were associated with a decrease in overall survival in patients with disseminated breast and pancreatic cancers.15 In addition, cancer patients with VTE presenting with the highest MP TF activity had a lower survival rate than did patients with low MP TF activity.111 Finally, we found a median survival time of only 98.5 days in a high MP TF activity group in comparison with 231 days for a low MP TF activity group in a cohort of 117 pancreatic and biliary cancer patients.114

Taken together, these studies strongly suggest that TF+ MPs and MP TF activity may have prognostic value in identifying cancer patients with increased risk of VTE. It should be noted that pancreatic cancer patients have higher levels of circulating TF+ MPs than do patients with other types of cancer, suggesting that TF+ MPs may not be a useful biomarker for thrombosis risk in all types of cancer. Indeed, cancer type was one factor that was used in a risk assessment score to predict thrombosis in cancer patients.116 Interestingly, a new clinical trial (Microparticle Thromboprophylaxis with Enoxaparin in Cancer or MicroTEC. Available at http://clinicaltrials.gov) will evaluate the benefits of prophylaxis with low molecular weight heparin in cancer patients with high levels of TF+ MPs in the prevention of cancer-induced thrombosis.117

Analysis of TF+ MPs in Hyperlipidemic Mice and in Patients with Cardiovascular Disease

Plaque disruption and subsequent arterial thrombosis is a major complication of atherosclerosis (termed atherothrom-
This results in acute vascular syndromes, such as myocardial infarction and stroke.\textsuperscript{118} Large amounts of TF are present in atherosclerotic plaques.\textsuperscript{119} Furthermore, TF expression increases with the progression of atherosclerotic plaques, and higher TF activity is observed in plaques with thrombi.\textsuperscript{120} Importantly, much of this TF is speculated to be in the form of TF\textsuperscript{+} MPs.\textsuperscript{2} The following sections will review studies on TF\textsuperscript{+} MPs in animal models of arterial thrombosis and in human atherothrombosis.

### Role of Hematopoietic Cell-Derived TF\textsuperscript{+} MPs in the Activation of Coagulation in Hyperlipidemic Mice

Hyperlipidemia results in the formation of oxidized low-density lipoproteins (oxLDLs). Indeed, patients with high levels of oxLDL autoantibodies have elevated levels of TF\textsuperscript{+} MPs derived from monocytes.\textsuperscript{121} We found that oxLDL induces TF expression in monocytes and the release of TF\textsuperscript{+} MPs (A. P. Owens and N. Mackman, unpublished data.

### Table. Circulating TF in Clinical Studies

<table>
<thead>
<tr>
<th>Disease State</th>
<th>Specific Condition</th>
<th>TF\textsuperscript{+} Flow Cytometry</th>
<th>MP TF Activity</th>
<th>Major Finding of Study</th>
<th>Reference No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cancer</td>
<td>Colorectal</td>
<td>Yes</td>
<td>No</td>
<td>Increased TF\textsuperscript{+} MPs in cancer vs healthy controls, correlated with D-dimer.</td>
<td>38</td>
</tr>
<tr>
<td>Cancer</td>
<td>Pancreatic and breast</td>
<td>Yes</td>
<td>Yes</td>
<td>Increased TF\textsuperscript{+} MPs and increase in MP TF activity in cancer vs controls. Increased MP TF activity associated with decreased survival.</td>
<td>15</td>
</tr>
<tr>
<td>Cancer</td>
<td>Multiple forms</td>
<td>No</td>
<td>Yes</td>
<td>Increased MP TF activity in metastatic cancer patients vs healthy controls.</td>
<td>136</td>
</tr>
<tr>
<td>Cancer</td>
<td>Pancreatic</td>
<td>No</td>
<td>Yes</td>
<td>Increasing MP TF activity and TF antigen predictive of VTE.</td>
<td>14</td>
</tr>
<tr>
<td>Cancer</td>
<td>Prostate</td>
<td>Yes</td>
<td>Yes\textsuperscript{a}</td>
<td>MP TF activity is increased in cancer vs controls and correlated with D-dimer.</td>
<td>112</td>
</tr>
<tr>
<td>Cancer</td>
<td>Multiple myeloma</td>
<td>No</td>
<td>Yes</td>
<td>MP TF activity is increased in cancer vs controls and reduced with chemotherapy.</td>
<td>35</td>
</tr>
<tr>
<td>Cancer</td>
<td>Multiple forms</td>
<td>Yes\textsuperscript{a}</td>
<td>No</td>
<td>Cancer patients with VTE had higher MP TF activity vs controls. Increased MP-TF activity resulted in decreased survival vs patients with low MP TF activity.</td>
<td>111</td>
</tr>
<tr>
<td>Cancer</td>
<td>Multiple forms</td>
<td>Yes\textsuperscript{a}</td>
<td>No</td>
<td>Increased TF\textsuperscript{+} MPs in cancer patients vs controls and are predictive of VTE.</td>
<td>108</td>
</tr>
<tr>
<td>Cancer</td>
<td>Pancreatobiliary</td>
<td>No</td>
<td>Yes</td>
<td>Increased MP TF activity in cancer patients and correlated to VTE.</td>
<td>114</td>
</tr>
<tr>
<td>Cancer</td>
<td>Multiple forms</td>
<td>No</td>
<td>Yes</td>
<td>Increased MP TF activity in patients with cancer and VTE vs cancer without VTE.</td>
<td>113</td>
</tr>
<tr>
<td>Cancer</td>
<td>Multiple forms</td>
<td>Yes</td>
<td>No</td>
<td>Increased TF\textsuperscript{+} MPs in cancer patients w/ and w/o VTE vs healthy controls.</td>
<td>109</td>
</tr>
<tr>
<td>ACS</td>
<td>MI</td>
<td>Yes</td>
<td>Yes\textsuperscript{c}</td>
<td>Decreased TFPI\textsuperscript{+} MPs and increased MP TF activity after thrombolysis.</td>
<td>132</td>
</tr>
<tr>
<td>ACS</td>
<td>MI</td>
<td>No</td>
<td>Yes</td>
<td>MP TF activity increased in patients with persistent occlusion vs controls.</td>
<td>133</td>
</tr>
<tr>
<td>ACS</td>
<td>MI</td>
<td>No</td>
<td>Yes</td>
<td>MP TF activity was increased in failed vs successful thrombolysis in patients.</td>
<td>134</td>
</tr>
<tr>
<td>ACS</td>
<td>MI</td>
<td>No</td>
<td>Yes\textsuperscript{c}</td>
<td>MP TF activity increased in lesion blood vs postangioplasty.</td>
<td>131</td>
</tr>
<tr>
<td>Diabetes</td>
<td>Type II diabetes</td>
<td>Yes</td>
<td>No</td>
<td>2× increased TF\textsuperscript{+} MPs in patients with type II diabetes vs healthy controls.</td>
<td>37</td>
</tr>
<tr>
<td>Diabetes</td>
<td>Type II diabetes</td>
<td>Yes</td>
<td>No</td>
<td>3× increased TF\textsuperscript{+} MPs in patients with type II diabetes vs healthy controls.</td>
<td>135</td>
</tr>
<tr>
<td>Sepsis</td>
<td>Meningococcal</td>
<td>Yes</td>
<td>No</td>
<td>Increased TF\textsuperscript{+} MPs in patients with meningococcal sepsis.</td>
<td>45</td>
</tr>
<tr>
<td>Endotoxemia</td>
<td>LPS administration</td>
<td>Yes</td>
<td>No</td>
<td>Increased TF\textsuperscript{+} MPs and MP TF activity in healthy volunteers given endotoxin.</td>
<td>13</td>
</tr>
<tr>
<td>Sickle Cell</td>
<td>Sickle cell disease</td>
<td>Yes</td>
<td>Yes\textsuperscript{a}</td>
<td>Increased TF\textsuperscript{+} MPs and MP TF activity in sickle cell patients vs controls.</td>
<td>46</td>
</tr>
</tbody>
</table>

ACS = acute coronary syndrome, MI = myocardial infarction, TF = tissue factor, MP = microparticles, VTE = venous thromboembolism, TFPI = tissue factor pathway inhibitor.

\textsuperscript{a}Microparticles assessed by flow impedance.

\textsuperscript{b}MP TF activity assessed by 1-stage clotting reaction with inclusion of anti-TF antibody.

\textsuperscript{c}Assay is considered controversial and may not be indicative of specific MP TF activity.
2010). Furthermore, hyperlipidemic mice have elevated levels of MP TF activity and TAT.122 Hyperlipidemic mice containing bone marrow from low-TF mice had decreased levels of MP TF activity, indicating that hematopoietic cells were the source of the TF \( ^+ \) MPs. These results suggest that TF \( ^+ \) MPs may enhance arterial thrombosis, for instance, after rupture of an atherosclerotic plaque. Indeed, previous studies have shown that hyperlipidemic mice had shorter occlusion times in the carotid artery model when compared with control mice.81,123,124 However, it is difficult to determine the contribution of elevated levels of TF \( ^+ \) MPs in models of arterial thrombosis because of the large amounts of TF in the vessel wall. Interestingly, we have found that hyperlipidemic mice have increased levels of fibrin accumulation in comparison with controls in a laser injury model of cremaster arterioles (A.P. Owens and F. Passam, unpublished data, 2010). Further experiments are required to show that this increase in fibrin is dependent on the elevated levels of TF \( ^+ \) MPs in the hyperlipidemic mice.

**Clinical Studies**

Leroyer and colleagues demonstrated that atherosclerotic plaques had 200-fold higher concentrations of leukocyte, erythrocyte, SMC, and endothelial cell–derived MPs in comparison with blood of the patients, which mainly consisted of platelet MPs.125 Importantly, more than 50% of the MPs isolated from the plaques were TF \( ^+ \) MPs. Another study found that 97% of the total MP procoagulant activity extracted from atherosclerotic plaques was due to TF.126 Moreover, the MPs isolated from the plaques were highly thrombogenic in comparison with the MPs isolated from the blood of the same patients. In a subsequent proteomics analysis of atherosclerotic plaque MPs, Mayr and colleagues demonstrated that 90% of the plaque-derived MPs were CD14\(^+\), indicating monocyte/macrophage origin.127 Finally, Bonderman and colleagues demonstrated increased TF activity associated with MPs when analyzing the scrapings from ex vivo endarterectomy samples.128 It is speculated that during plaque rupture, these TF \( ^+ \) MPs initiate thrombosis.

Soejima and colleagues were the first to describe increased levels of plasma TF antigen patients with unstable angina.129 Furthermore, Mallat and colleagues found an increase in circulating procoagulant MPs in patients presenting with acute coronary syndrome (unstable angina and myocardial infarction) versus those with stable angina and noncoronary patients (Table).130 Moreover, Morel and associates demonstrated an increase in procoagulant monocyte and endothelial cell–derived MPs in patients undergoing angioplasty.131 Steppich and colleagues examined patients with acute myocardial infarction (AMI), randomly assigned to either intravenous thrombolysis or coronary stenting.132 The numbers of TF \( ^+ \) MPs were similar in both groups of patients, although the thrombolysis group had a decrease in the number of TF \( ^+ \) MPs that contained for TFPI.

Huisse and colleagues prospectively enrolled 123 patients with AMI and demonstrated that MP TF activity was increased in the patients with persistent occlusion versus healthy controls.133 In a follow-up study, they found that patients who failed to achieve thrombolysis had significantly elevated MP TF activity.134 These results suggest that the failure to resolve thrombi may be due to resupply of TF \( ^+ \) MPs from the circulation to the thrombus. Morel and colleagues demonstrated an increase in MP TF activity in blood collected at the site of thrombus and at the site of thrombus postangioplasty, both of which were significantly increased over levels collected in the femoral blood.131

These results indicate that levels of TF \( ^+ \) MPs, most likely derived from activated monocytes and macrophages, are elevated in patients with cardiovascular disease. However, it is unclear whether levels of TF \( ^+ \) MPs will have a value in predicting future thrombotic events, because the major source of TF in these cases is the plaque itself. Nevertheless, further studies are needed to determine whether acute coronary syndrome patients with elevated levels of TF \( ^+ \) MPs have a worse prognosis.

**Other Diseases with Elevated Levels of TF \( ^+ \) MPs**

Elevated levels of circulating TF \( ^+ \) MPs are observed in a variety of disease states, including sepsis, diabetes, and sickle cell disease (Table).34,37,46,131,135,136 It is speculated that increased levels of TF \( ^+ \) MPs are likely to contribute to thrombosis. As an example, Shet and colleagues found an increase in monocyte and endothelial cell–derived TF \( ^+ \) MPs in sickle cell patients in comparison with healthy controls. These MPs had functional TF activity that was associated with enhanced activation of coagulation in sickle cell patients with steady state disease or undergoing crisis. One study found that patients presenting with acute VTE in the absence of malignant cancer did not have elevated levels of TF \( ^+ \) MPs, whereas another study demonstrated that patients with unprovoked VTE had higher TF \( ^+ \) MPs than did control patients.109,117 Cumulatively, these data demonstrate an increase in TF \( ^+ \) MPs and MP TF activity in a variety of disease states. It is also suggestive that TF \( ^+ \) MPs may serve as prognostic indicators for the risk of thrombotic events and potentially for survival. Further prospective trials are needed to determine if TF \( ^+ \) MPs are a valuable biomarker in diseases associated with thrombosis.

**Conclusion**

There are numerous reports analyzing levels of PS \( ^+ \) and TF \( ^+ \) MPs in health and disease. However, methods of MP analysis are not optimal, and this has led to much variation between studies. Development of machines that can more accurately quantify levels of PS \( ^+ \) and TF \( ^+ \) MPs in plasma and standardization of functional clotting assays will help to advance the field. At present, it is unclear whether PS \( ^+ \) MPs or TF \( ^+ \) MPs play a role in hemostasis. In contrast, several studies have shown an association between tumor-derived TF \( ^+ \) MPs and VTE in cancer patients, suggesting that these TF \( ^+ \) MPs trigger venous thrombosis. Therefore, TF \( ^+ \) MPs may be a useful biomarker to identify cancer patients, and possibly other patients, who have an increased risk of venous thrombosis. The elevated levels of monocyte-derived TF \( ^+ \) MPs observed in hyperlipidemia patients may also contribute to arterial thrombosis after rupture of atherosclerotic plaques. Further studies are needed to examine whether and how
different forms of MPs play a role in hemostasis and thrombosis.

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Disclosures
None.

References


100. Manly DA, Boles J, Mackman N. Role of tissue factor in venous thrombosis.


Microparticles in Hemostasis and Thrombosis
A. Phillip Owens III and Nigel Mackman

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