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2011 Phys. Biol. 8 066005

(http://iopscience.iop.org/1478-3975/8/6/066005)

Phys. Biol. **8** (2011) 066005 (7pp) doi:10.1088/1478-3975/8/6/066005

The role of carrier number on the procoagulant activity of tissue factor in blood and plasma

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Received 13 June 2011
Accepted for publication 27 September 2011
Published 2 November 2011
Online at stacks.iop.org/PhysBio/8/066005

Abstract
Tissue factor (TF) is a transmembrane glycoprotein cofactor of activated blood coagulation factor VII (FVIIa) that is required for hemostatic thrombin generation at sites of blood vessel injury. Membrane-associated TF detected in circulating blood of healthy subjects, referred to as intravascular or circulating TF has been shown to contribute to experimental thrombus propagation at sites of localized vessel injury. Certain disease states, such as metastatic cancer, are associated with increased levels of intravascular TF and an elevated risk of venous thromboembolism. However, the physiological relevance of circulating TF to hemostasis or thrombosis, as well as cancer metastasis, is ill-defined. This study was designed to assess whether the spatial separation of intravascular TF carriers in blood, demonstrated with TF-inducible human monocytic cell line U937 or TF-coated polymer microspheres, affected procoagulant activity and hence thrombogenic potential. Experiments were performed to characterize the effects of TF-carrier number on the kinetics of clot formation in both open and closed systems. The procoagulant activity of TF carriers was found to correlate with spatial separation in both closed, well-mixed systems and open, flowing systems. TF carriers enhanced the amidolytic activity of FVIIa toward the chromogenic substrate, S-2366, as a function of carrier count. These results suggest that TF-initiated coagulation by circulating TF is kinetically limited by mass transport of TF-dependent coagulation factors to the TF-bearing surface, a constraint that may be unique to circulating TF. Spatial separation of circulating TF carriers is therefore a critical determinant of the procoagulant activity of circulating TF.

Nomenclature

TF:	tissue factor
FVII, FX, FXa:	coagulation factor VII, coagulation factor X, activated factor X, respectively
FBS:	fetal bovine serum
BSA:	bovine serum albumin
PBS:	phosphate buffered saline
HBSS:	Hank's balanced salt solution
PRP, PPP:	platelet-rich plasma, platelet-poor plasma, respectively
LPS:	lipopolysaccharide
SEM:	standard error of the mean

1. Introduction

The increased incidence of venous thromboembolism for metastatic cancer patients is a well-known phenomenon first

described nearly two centuries ago [1, 2] and embodies the second leading cause of death in cancer patients. The association between metastatic cancer and coagulation suggests that metastasizing cancer cells are not inert to the blood environment.

Cancer patients who suffer thromboses have demonstrated elevated levels of circulating TF in the form of TF-bearing microparticles [3], while cancer cell lines in culture have been shown to surface express TF [4]. TF, the physiologic initiator of coagulation, complexes with FVII to activate coagulation factors IX (FIX) and X (FX). The surface-bound nature of TF [5], its requirement for phospholipids in order to initiate coagulation [6] and the need for FVII to combine with TF to achieve physiologically relevant reaction rates [6] imply that the initiation of coagulation by TF is a surface phenomenon. Kinetics of TF-initiated enzyme activation have been found to depend upon physicochemical characteristics of the TF-bearing surface and its interface with blood [7–9]. The discovery of intravascular, circulating TF [10–15] has brought anew the importance of understanding kinetic limitations on TF-initiated thrombin generation, as the proximity and mixing of circulating TF to blood is different in many aspects compared to TF locally exposed on the vascular wall at sites of disease or injury. *In vivo* [16] and *in vitro* [17], circulating TF has been shown to be active in initiating coagulation and indeed contribute to experimental thrombosis at sites of vessel injury [18, 19]. However, healthy subjects have been shown to have a basal level of circulating TF without evidence for pathological initiation of coagulation [17, 20]. Conversely, induction of TF-bearing microparticles restored hemostasis in a mouse model of hemophilia [21]. Therefore, the circumstances that dictate whether circulating TF contributes to thrombotic or hemorrhagic coagulopathy versus normal hemostasis have not been reconciled.

Studies on the ability of wall-bound TF to initiate coagulation have shown that the critical TF surface density to result in surface deposition of fibrin is largely dependent upon flow parameters [8]. Generation of FXa was shown to depend upon the presence of phospholipids and FVII levels in an *in vitro* system of TF surface-catalyzed coagulation [7]. Studies with lymphoid cell lines support the notion that the membrane environment, rather than TF exposure, is the dominating factor in determining procoagulant activity of TF [9]. How TF influences cancer cell procoagulant activity for vessel-wall adherent versus circulating cancer cells has not been determined. The presence of convection mass transport from blood flowing past an adherent TF-expressing cancer cell presents the potential for different procoagulant activities as compared to a circulating cell, which experiences significantly less relative flow of blood across its surface. Therefore, it is prudent to determine the TF procoagulant activity for circulating cells or microparticles under physiological conditions of flow.

This study was designed to characterize how the procoagulant phenotype of intravascular TF is dependent upon the spatial distribution of circulating TF. We hypothesize that by increasing the proximity of TF carriers to bulk plasma, through increasing the distribution of TF-bearing surfaces throughout blood, we will increase the procoagulant activity of circulating TF by reducing the average distance between coagulation factors and a TF surface. We characterized the procoagulant and prothrombotic phenotype of TF carriers, consisting of the TF-inducible monocytic cell line U937 and TF-coated polymer microspheres, in both open and closed systems, and in both static and flow conditions. Our results demonstrate that for circulating TF, spatial distribution plays a role in determining coagulation initiation and propagation kinetics, and suggest that circulating TF procoagulant and prothrombotic activity may be independent of whole blood TF concentration.

2. Methods

2.1. Reagents

All reagents were purchased from Sigma-Aldrich (St Louis, MO) or previously described sources [22] unless otherwise specified. Monoclonal antibodies (mAbs) to factor XI were generated as previously described [18]. Neutralizing anti-TF antibodies (clone D3H44) were obtained from Genentech (South San Francisco, CA) and fluorescein isothiocyanate (FITC)-conjugated anti-TF antibodies were obtained from Lifespan Biosciences (Seattle, WA). Recombinant, lipidated TF (Dade® Innovin®) was purchased from Siemens Healthcare Diagnostics (Deerfield, IL). Fibrillar equine collagen was from Chrono-log (Havertown, PA). Biowhittaker® Premium FBS was from Lonza (Basel, Switzerland).

2.2. Blood donations

All donations from healthy human subjects were obtained in accordance with Oregon Health and Science University IRB approval. Blood was collected by antecubital venipuncture into a one-tenth volume 3.8% sodium citrate for occlusive thrombus assays or 3.2% sodium citrate for coagulation and enzyme generation assays. PPP for coagulation studies was prepared by centrifugation of citrated blood at 2150 *g* for 10 min. PRP was decanted from the spun blood, pooled with PRP from two other donors and subjected to centrifugation at 2150 *g* for 10 min. PPP was then collected by decanting off the supernatant. Aliquots of PPP were immediately frozen at −80 °C until use.

2.3. Human monocytic U937 cell culture

U937 cells were purchased from ATCC (Manassas, VA). Cells were cultured in non-treated T25 flasks in a medium consisting of RPMI 1640 containing 10% FBS and 1× penicillin–streptomycin and kept in an incubator at 37 °C containing 5% CO_2. The culture medium was supplemented weekly with L-glutamine. Flasks were seeded at a concentration of 2×10^5 mL^{-1} and kept below 2×10^6 mL^{-1} with viability maintained above 90%. All experiments were performed between passages 4 and 7.

2.4. U937 induction of TF

U937 cells were incubated with 50 μg mL^{-1} anti-TF antibody to minimize the influence of constitutively expressed TF. Cells were then split and resuspended in the normal culture media or media containing 10 μg mL^{-1} LPS and placed in an incubator for 24 h. Then, cells were pelleted by centrifugation at 130 *g* for 10 min and resuspended in HBSS. Suspensions were diluted from a concentration of 1×10^7 to 1×10^2 mL^{-1} with HBSS as determined by a hemocytometer.

2.5. Microsphere coating

Polymeric microspheres (diameter = 9.86 μm) that approximated the size of human monocytes (diameter $\sim$ 7–10 μm) were purchased from Bangs Laboratories (Fishers, IN). Stock solution microsphere concentrations were created by diluting in H_2O (resistivity = 18.2 MΩ cm^{-1}) and counted with a hemocytometer. One million microspheres were then dispensed into a 1.7 mL vial. TF coating solutions were prepared by diluting TF stock to 1 nM in H_2O. 1 mL of coating solution was added to the microspheres and allowed to coat for 60 min at room temperature. Coated microspheres were pelleted by centrifugation at 16 100 *g* for 10 min and surface blocked with 0.5% denatured-filtered BSA in PBS. Coated and blocked microspheres were pelleted and resuspended in HBSS.

2.6. Flow cytometry analysis

One million LPS-stimulated U937 cells or TF-coated microspheres were washed with HBSS prior to incubation with a FITC-conjugated anti-TF antibody (50 μg mL^{-1}) for 30 min at room temperature. Following labeling, TF carriers were washed with HBSS and next analyzed on a FACSCalibur flow cytometer with CellQuest acquisition and analysis software (Becton Dickinson, Franklin Lakes, NJ). Unlabeled cells served as negative controls.

2.7. Clotting time determination

TF carriers were suspended in HBSS and carrier density counted with a hemocytometer. Suspension concentrations were then diluted from 1×10^6 to 1×10^2 TF carriers mL^{-1}. 50 μL of carrier suspension was added to 50 μL of PPP and allowed to mix for 180 s at 37 °C, after which 50 μL of 25 mM $CaCl_2$ in H_2O was added and the clotting time recorded on a KC4 Coagulation Analyzer (Trinity Biotech, Bray, Co. Wicklow, Ireland). To determine the procoagulant mechanism of microspheres and U937 cells, anti-FXI mAbs were added to PPP and allowed to mix for 5 min at 37 °C prior to addition of microsphere or cell suspensions and the clotting time recorded. Anti-TF antibodies were mixed with the carrier suspensions for 5 min at 37 °C before mixing with the PPP, and the resultant clotting time recorded. Reported values represent the average value for a minimum of three experiments.

2.8. Occlusive thrombus assay

Ex vivo occlusive thrombus assay was performed as previously described [19]. Briefly, glass capillary tubes (2.0 mm $\times$ 0.2 mm, VitroCom, Mountain Lakes, NJ) were coated with 100 μg mL^{-1} equine fibrillar collagen in 10 mM acetic acid for 60 min at room temperature while rotating on a carousel. Next, tubes were washed with PBS and surface-blocked with 0.5% denatured-filtered BSA in PBS for 60 min at room temperature while rotating on a carousel. Blocked tubes were then washed, and one end fitted with a 1 cm length of 0.40″ internal diameter silicone tubing and attached to a suspended 3 mL syringe while submerging the other end in PBS. TF carrier suspensions were mixed with citrated blood and recalcified immediately before subjecting them to flow. The time for the blood solutions to occlude flow in the collagen-coated capillary was recorded as the time to occlusion as described previously [4].

2.9. Enzyme generation assay

15 μL of 4 mM S-2366 substrate (Chromogenix, Milan, Italy) was combined with 50 μL of PPP and dispensed into individual wells of a 96-well plate. Next, 50 μL of TF-coated microsphere suspensions ranging from a concentration of 1×10^6 to 1×10^3 mL^{-1} were added to the wells and allowed to mix for 15 min at 37 °C. Then, 50 μL of 25 mM $CaCl_2$ in H_20 was added immediately followed by measurement of absorbance of 405 nm wavelength light at 22 °C from each well at 60 s intervals for 1 h in a spectrophotometer (Tecan, Mannedorf, Switzerland). Absorbance data exhibited a sigmoid relationship with time. The data were analyzed by first normalizing by the maximum and subtracting the baseline absorbances. Next, the initiation lag was recorded as the time corresponding to the first measurable increase in absorbance. Then, the molar equivalent of S-2366 added to the plasma (600×10^{-12} moles) was divided by the difference between the time points corresponding to 0% and 100% absorbance through extrapolation of the slope at 50% absorbance.

2.10. Spatial separation calculation

The average distance between TF carriers in suspension, referred to as spatial separation, was obtained by calculating the cubic root of the volume of liquid divided by the number of TF carriers added. This approach assumes that TF carriers are uniformly distributed in suspension.

2.11. Statistical analysis

Data are presented as mean $\pm$ standard error (SEM). Statistical significance between means was determined with the one-tailed paired Student's *t*-test utilizing Bonferroni's correction. Significance for all statistical tests required $P < 0.05$.

3. Results

3.1. Monocytic cell-line U937 and TF-coated polymer microspheres portray surface-bound TF

We initially determined the surface expression of TF on U937 cells and TF-coated microspheres. As shown in figure 1, flow

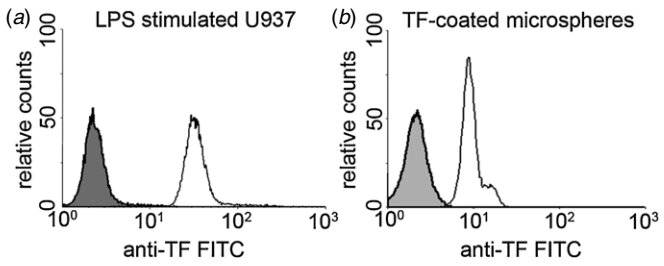

Figure 1. Characterization of TF expression on LPS-stimulated U937 cells and TF-coated polymer microspheres. Cultured U937 cells that were stimulated with 10 μg mL^{-1} LPS for 24 h or TF-coated microspheres (10^6 mL^{-1}) were labeled with a FITC-conjugated anti-TF antibody (50 μg mL^{-1}) and analyzed by flow cytometry. The shaded curves represent background fluorescence of cells or beads; white curves represent a shift in fluorescence in the presence of the anti-TF antibody. The representative curves from three to four independent experiments are shown.

cytometric analysis showed that both TF carriers portrayed TF on their surface. Unlabeled U937 cells resulted in a mean fluorescence of 3.1 ± 0.0 (n = 3), while FITC-conjugated anti-TF-antibody-labeled cells yielded a mean fluorescence of 130.1 ± 2.1 (n = 3). Non-stimulated U937 cells yielded a mean fluorescence of 109.3 ± 20.0 (n = 3). Unlabeled TF-coated microspheres yielded a mean fluorescence of 2.8 ± 0 .0 (n = 4), while FITC-conjugated anti-TF-antibody-labeled TF-coated microspheres yielded a mean fluorescence of 21.9 ± 0.5 (n = 4). BSA-coated control microspheres yielded a mean fluorescence of 5.9 ± 0.7 (n = 4).

3.2. Monocytic cell line-derived TF is procoagulant in a carrier number- and TF-dependent manner

To verify that our assays were sensitive to hematopoietic TF, the ability of TF-expressing U937 cell suspensions to coagulate plasma was assessed. As shown in figure 2(*a*), our results indicate that LPS-stimulated cells had higher procoagulant activity than non-stimulated U937 cells. The clotting times decreased with increasing counts of U937 cells from 1 × 10^4 to 1 × 10^6 mL^{-1} (figure 2(*b*)), and their ability to coagulate plasma was abrogated by the addition of an anti-TF antibody (figure 2(*a*)) The clotting time correlated linearly with the spatial separation of U937 cells (figure 2(*c*), R^2 = 0.9949). No significant effect on clotting time was observed for U937 counts below 1 × 10^5 mL^{-1}.

3.3. Synthetic TF carriers are procoagulant in a carrier number- and TF-dependent manner

We next investigated the ability of TF-coated microspheres to potentiate coagulation. Our data show that the addition of 1 × 10^6 mL^{-1} TF-coated microspheres dramatically reduced clotting times in a TF-dependent manner (figure 3(*a*)). Clotting times were unaffected by the blockade of the contact pathway with the anti-FXI mAbs, 14E11 or 1A6, unless the extrinsic pathway was blocked concomitantly (figure 3(*a*)). TF carrier suspensions from 1 × 10^2 to 1 × 10^6 mL^{-1} yielded clotting times that varied with the carrier count (figure 3(*b*)). Clotting time correlated with the spatial separation of TF-coated microspheres (figure 3(*c*), R^2 = 0.9992).

3.4. TF carriers promote occlusive thrombus formation in a TF- and carrier number-dependent manner

Experiments were designed to determine the role of carrier count on the prothrombotic activity of TF carriers in a flowing, open, whole blood assay by measuring the time required for occlusion of flow. Addition of 1 × 10^6 mL^{-1} TF-coated microspheres significantly reduced the time to occlusion in a TF-dependent manner (figure 4(*a*)). The prothrombotic activity of TF-coated microspheres was abrogated by the addition of an anti-TF antibody (figure 4(*a*)). Blockade of the contact pathway with 14E11 did not influence time to occlusion unless an anti-TF antibody was also included. TF-coated microspheres reduced the time to occlusion in a carrier number-dependent manner (figure 4(*b*)). Time to occlusion correlated with spatial separation of TF-coated microspheres (figure 4(*c*), R^2 = 0.9909).

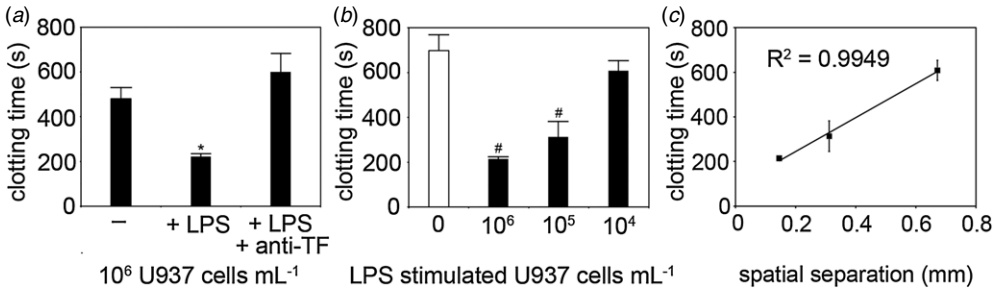

Figure 2. Characterization of the procoagulant activity of monocytic cells in a closed system. U937 cells were procoagulant in a TF-dependent (*a*) and cell-concentration-dependent manner (*b*) that correlated with the spatial separation of TF surfaces (*c*). Human sodium citrate-anticoagulated plasma was pretreated with vehicle (-) or a neutralizing antibody to TF (anti-TF, 20 μg mL^{-1}) prior to addition of cultured U937 cells (10^4–10^6 mL^{-1}) for 3 min at 37 °C. U937 cells were stimulated with LPS (10 μg ml^{-1} LPS) for 24 h. Coagulation of plasma was initiated by recalcification using 7.6 mM $CaCl_2$ (final concentration) and clotting times were recorded on a coagulometer. Data are reported as mean ± SEM, from—three to six experiments. $^*P < 0.05$ versus non-LPS stimulated cells. $\#P < 0.05$ versus the absence of cells.

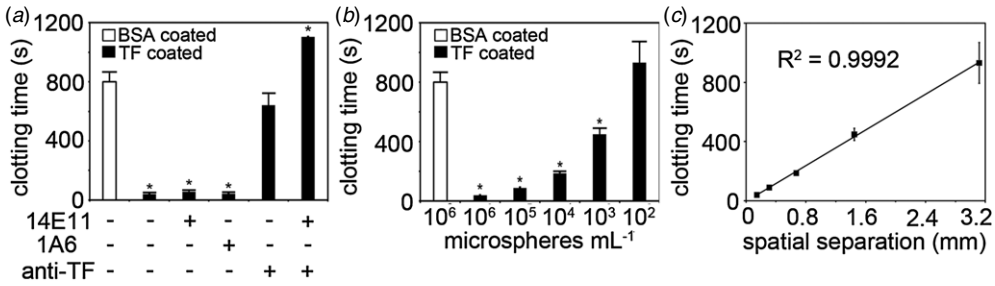

Figure 3. Characterization of the procoagulant activity of TF microspheres in a closed system. TF-coated polymeric microspheres were procoagulant in a TF- (*a*) and carrier-concentration-dependent manner (*b*) that correlated with spatial separation of TF surfaces (*c*). Human sodium citrate-anticoagulated plasma was pretreated with neutralizing antibodies to TF (anti-TF, 50 μg mL^{-1}) and FXI (14E11 or 1A6, 10 μg mL^{-1}) prior to addition of BSA-coated (white bars) or TF-coated (black bars) microspheres (10^2–10^6 mL^{-1}) for 3 min at 37 °C. Data in (*a*) were obtained with microsphere additions of 10^6 mL^{-1}. Coagulation of plasma was initiated by recalcification using 7.6 mM $CaCl_2$ (final concentration) and clotting times were recorded on a coagulometer. Data are reported as mean ± SEM, from three experiments. $^*P <$ 0.05 versus BSA-coated microspheres.

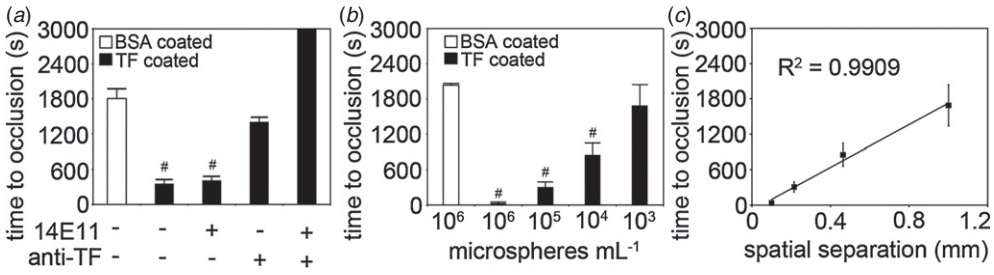

Figure 4. Characterization of prothrombotic activity of TF microspheres in an open system. TF-coated polymeric microspheres were prothrombotic in a TF-(*a*) and carrier-concentration-dependent manner (*b*) that correlated with spatial separation (*c*) when added to recalcified whole blood in an *ex vivo* occlusive thrombus assay. The procoagulant activity of 10^5 mL^{-1} TF-coated microspheres was abrogated with an anti-TF antibody. Blockade of the contact pathway of coagulation with 14E11 (anti-FXI) had no effect on time to occlusion for TF microsphere suspensions in the absence of anti-TF antibodies (*c*). TF-coated microspheres (10^3–10^6 mL^{-1} final concentration) were added to human sodium citrate-anticoagulated blood prior to recalcification using 7.6 mM $CaCl_2$ (final concentration). Occlusive thrombus formation was initiated by flowing the pretreated blood at a constant pressure through a collagen-coated capillary tube and the time to occlusion was recorded when flow ceased. Data are reported as mean ± SEM, from three experiments. $^*P < 0.05$ versus non-LPS stimulated cells. #$P < 0.05$ versus BSA-coated microspheres.

3.5. Enzymatic initiation time and reaction rate are carrier number dependent

Experiments were designed to investigate whether the time required for assembly of active enzyme complexes upon the TF carriers or the reaction rates of assembled complexes were dictated by carrier number. Cleavage of the chromogenic substrate S-2366 was performed to measure the initiation time lag (initiation time) for activated enzyme to form as well as the rate at which formed active enzymes cleaved the substrate (rate). The initiation time decreased as the TF-coated microsphere carrier count increased (figure 5(*a*)). The rate of substrate cleavage, on the other hand, was found to increase with increases in carrier count (figure 5(*b*)). The initiation time correlated with spatial separation (figure 5(*c*), $R^2 = 0.9643$), whereas enzyme reaction rates correlated with inverse spatial separation (figure 5(*d*), $R^2 = 0.9927$).

4. Discussion

This and other studies have shown that the addition of TF-expressing cells to plasma results in shortened clotting times in a cell concentration manner. The ability of TF-expressing cells to initiate coagulation has been shown to depend on the physicochemical properties of the cell–plasma interface. In other words, the mere presence of TF does not dictate its ability to promote coagulation, even though the mechanism of coagulation initiation is TF dependent. In this study, we implemented a model system utilizing a single, fully active form of TF (thromboplastin) adsorbed onto the surfaces of polymer microspheres to determine if the spatial separation of TF surfaces would impact its procoagulant activity. Our data show that the clotting time linearly correlated with the spatial separation of TF carriers in both well-mixed, closed-system plasma coagulation assays (figures 2(*c*) and 3(*c*), $R^2 = 0.9949$ and 0.9992, respectively). Additionally, the time to occlusion linearly correlated with the spatial separation of TF carriers in a flowing, open-system whole blood occlusive thrombus assay (figure 4(*c*), $R^2 = 0.9909$). In a purified system, the initiation time correlated with spatial separation (figure 5(*c*), $R^2 = 0.9643$), while the enzyme reaction rate showed an inverse correlation with spatial separation (figure 5(*d*), $R^2 = 0.9927$).

We found that the spatial separation of TF surfaces, as calculated through carrier number, strongly correlates with procoagulant and prothrombotic activity. These results

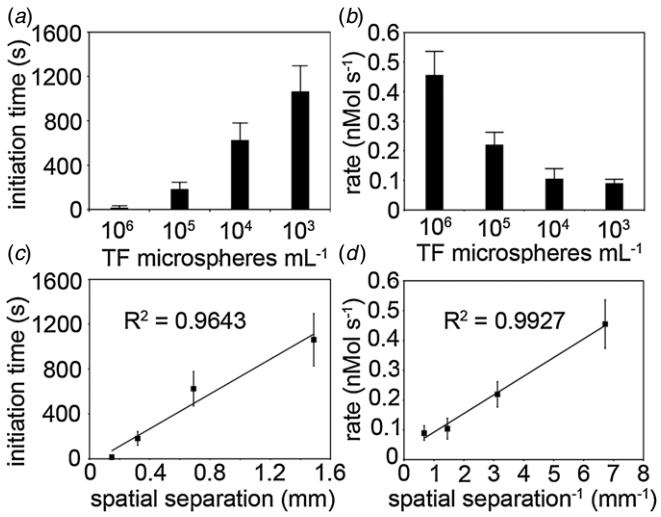

Figure 5. Characterization of enzyme activation kinetics initiated with TF microspheres. TF-coated polymeric microspheres promoted assembly of active enzyme complex in a carrier-concentration-dependent manner (*a*). The rate of substrate cleavage was dependent upon carrier count (*b*). Enzyme initiation time yielded a direct relationship with the calculated spatial separation between TF microspheres in suspension (*c*). Enzyme reaction rate yielded an inverse relationship with spatial separation for TF microspheres (*d*). Human sodium citrate-anticoagulated plasma was pretreated with 15 μL of S-2366 before addition of 50 μL of TF microspheres (10^3–10^6 mL^{-1}) and recalcification using 7.6 mM $CaCl_2$ (final concentration). Absorbance of 405 nm wavelength light was recorded with a spectrophotometer at 1 min intervals for 60 min. Data are reported as mean ± SEM, from —four to seven experiments.

suggest that mass transport may be a prominent kinetic limitation for intravascular TF under steady-state conditions and supports the notion that procoagulant activity of circulating TF is kinetically constrained by enzyme complex formation on the TF-bearing surfaces, which itself has been shown to depend upon the transport of coagulation factors from bulk to the TF-bearing surface [23, 24]. Immobilized TF has been shown to transition from diffusion-limited to convection-assisted reaction kinetics above wall shear rates of 120 s^{-1} [7]. A transition from diffusion-limited reaction kinetics for circulating TF (lower procoagulant activity) to convection-assisted kinetics for immobilized TF (higher procoagulant activity) may explain how TF can circulate without initiating clinically evident coagulation, yet contribute to thrombus propagation when incorporated into the growing thrombus. In essence, the procoagulant activity of TF may be regulated independent of whole blood TF concentration, but rather by the distribution of TF within the whole blood as well as the relative flow of blood past the TF-bearing surface. Thus, our data suggest the potential for circulating TF carriers to increase procoagulant activity based solely on changes in spatial separation. Further, our results suggest that a TF-expressing cell, such as a metastatic cancer cell, may yield different procoagulant abilities based on its relative mobility within the vasculature. Specifically, a vessel wall adherent cell, as may be encountered for an intravasating or extravasating cell, would yield a procoagulant activity that is dictated by convection-mediated transport, while a circulating cell's procoagulant activity is dictated by diffusion-mediated transport. Additionally, these results also support the idea that turbulent flow would increase circulating TF procoagulant activity through enhanced substrate mass transport to the TF carrier surface through convection-assisted mixing. Anecdotally, regions of turbulent flow due to branching vessels in a baboon sepsis model were shown to undergo increases in TF-dependent coagulation [25].

In suspension, increasing the TF carrier count, and thus the proximity of reacting TF surface to soluble substrate, increases the rate of formation of active enzyme complexes per unit volume. Increases in TF carrier count in blood, such as due to an increase in the number of circulating TF-bearing microparticles, could promote thrombosis. Here, in its simplest form, a parent cell sheds microparticles. These microparticles, being composed largely of the parent cell membrane, would not alter the concentration of circulating TF within the blood. However, the TF carrier count per unit volume of blood would be dramatically increased, resulting in plasma coagulation factors having greater access to TF surfaces, hastening their conversion to active enzyme. This may elevate the procoagulant activity of circulating TF in the blood. For instance, the induction of microparticles by monocytes in models of endotoxemia [11] and in patients with paroxysmal nocturnal hemoglobinuria [26] has been reported, both of which are associated with thrombotic complications. Similarly, a link between intravascular TF and cancer-cell-derived microparticles has been observed [27], perhaps explaining why cancer patients experience elevated rates of venous thromboembolism. Our results suggest a novel TF procoagulant activity-regulating mechanism, independent of the concentration of TF, which may contribute to understanding the physiological significance and clinical relevance of intravascular TF.

The case for spatial separation dictating the procoagulant activity of intravascular TF may highlight a novel therapeutic strategy for coagulopathies associated with intravascular TF. While reducing expression of TF could reduce the thrombogenecity of a microparticle, such as by inhibiting TF expression on LPS-induced monocytic cell lines by activated protein C [28], it is possible that inhibiting the formation of microparticles from the TF-expressing parent cell could also be effective at preventing thrombosis. For instance, IL-10 administration was found to reduce thrombin generation of LPS-stimulated monocytes, which resulted in concomitant reductions in microparticle formation as well as TF expression [29]. Moreover, blockade of the leukocyte receptor, PSGL-1, has been shown to reduce leukocyte-derived microparticles [21], while PSGL-1$^{-/-}$ mice were unable to incorporate circulating TF into thrombi at sites of vessel injury [18]. Whether reduction in circulating TF activity is due to inhibition of TF expression or microparticle generation is difficult to know, as inhibition of one is often linked with inhibition of the other. To our knowledge, isolation of microparticle generation from TF expression has not been performed or quantified.

This study aimed to determine the impact of TF carrier count on the procoagulant activity of TF. A direct relationship

of clotting time, time to occlusion and enzyme initiation time was seen with spatial separation, while enzyme reaction rates yielded an inverse correlation with spatial separation, a direct function of carrier number. These findings were evaluated in light of mass transport limitations, which suggest for the first time that the determinants of prothrombotic intravascular TF activity, hence the risk of developing pathological clots or occlusive thrombi, include the TF carrier count. The paucity of thrombotic events in the presence of intravascular TF may be explained by kinetically constrained mass transport of coagulation factors to the TF carrier. Such a limitation may be overcome to achieve clinical relevance when the carrier burden reaches a critical level or when a transition from diffusion-limited to convection-assisted reaction kinetics occurs when circulating TF incorporates into a growing thrombus or a circulating cell adheres to a vessel wall. It is interesting to note that these are the physical parameters, independent of the changes in a patient's coagulation profile and whole blood TF concentration, which may influence the procoagulant activity and clinical relevance of circulating TF.

Acknowledgments

This work was supported in part by the National Institute of Health (U54CA143906, R01HL038779) and the Oregon Health and Science University School of Medicine MD/PhD program. The content is solely the responsibility of the authors and does not necessarily represent the official view of the funding agencies.

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