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ORIGINAL ARTICLE

A rapid, sensitive, and specific assay to measure TF activity based on chromogenic determination of thrombin generation

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Abstract

Background: Most tissue factor (TF) activity assays are based on measurement of factor X (FX) activation by TF in the presence of factor VII (FVII)/FVIIa. This requires long incubation, which may result in TF-independent activity of FX and inaccurate measurement of TF activity.

Aim: To develop a sensitive and specific TF activity assay, which does not register a non-specific TF activity, using commercial coagulation factors.

Methods: Tissue factor activity was measured based on the ability of TF to accelerate the activation of FX by FVIIa in the presence of factor V (FV)/Va, prothrombin, and phospholipids. Following 4 min incubation at 37°C, TF activity was quantified in test samples of different nature by thrombin generation using a chromogenic substrate.

Results: The TF activity assay proved high sensitivity (low fM range) and specificity, assessed by neutralization of TF activity by anti-TF antibody and the use of FVIIai. TF activity was detected in extracellular vesicles (EVs) derived from HAP1-TF+cells, while no activity was measured in EVs from HAP1-TF/KO cells. The assay was applicable for measurement of TF activity on the surface of live endothelial cells and monocytes activated *in vitro*, and cell lysates. Infusion of low dose lipopolysaccharide (2 ng/kg bodyweight endotoxin) caused a transient 8-fold increase (peaked at 4 h) in TF activity in EVs isolated from plasma of healthy volunteers.

Conclusion: Our assay provides a fast, sensitive, and specific measurement of TF activity. It reliably quantifies TF activity on cell surface, cell lysate, and isolated EVs. The assay can be used for laboratory and clinical research.

KEYWORDS
coagulation factor, endotoxemia, extracellular vesicles, phospholipids, tissue factor

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1 | INTRODUCTION

Tissue factor (TF) is a transmembrane glycoprotein that serves as the primary activator of blood coagulation *in vivo*,[1] through binding to factor VII/VIIa (FVII/VIIa) with subsequent limited proteolysis of factor IX (FIX) and factor X (FX), ultimately leading to thrombin generation.[2] Under physiological conditions, TF is absent or expressed at very low levels in cells that come in direct contact with circulating blood. Monocytes, the major source of TF in blood,[3] are important regulators of blood thrombogenicity through expression of TF on their surface and shedding of procoagulant extracellular vesicles (EVs) under various pathological conditions.[4] EVs are released from activated cells and exert their procoagulant function by expression of phosphatidylserine (PS) at the outer leaflet of the membrane[5,6] and thereby facilitate the assembly of coagulation FVII, FIX, FX, and prothrombin (FII)[7] and accelerate the activity of the TF:FVIIa complex by several orders of magnitude.[8] In addition, EVs display TF under pathological conditions such as certain cancer types,[9,10] sepsis,[11] and arterial and venous thromboembolic diseases.[12]

Reliable and accurate measurement of TF expression (as an antigen), or its activity in cells and EVs, is a prerequisite to explore the role of TF in normal hemostasis and the pathogenesis of various conditions, and thromboembolic diseases in particular. Currently, the activity measurements dominate over the antigen assays, as they are more sensitive and reliable than antigen-based assays.[13,14] The lack of a standardized TF activity assay along with the widespread use of different in-house assays with low sensitivity and poor specificity[14,15] have led to controversy on the localization and expression of TF, and its importance in the development of thrombosis.[16] Most activity-based assays quantify TF using a chromogenic substrate to detect FXa generation following incubation of FVIIa and FX with the TF-test-sample in the presence and absence of anti-TF antibody.[17] The drawback of such assays is the requirement of long incubation of 60 to 120 min to allow measurable concentrations of FXa due to the high Km (Michaelis-Menten constant) of FXa (0.1 mol/L) to cleave its substrate. The outgrowth of this long incubation may lead to a generation of FXa that occurs independent of TF via different pathways, and erroneously translate into too high TF activity values. First, the activation of FX by FVII/FVIIa/TF complex is dependent on the presence of phospholipids.[18] Suboptimal concentrations of phospholipids used in FXa-based TF activity assays result in slow activation of FX, and hence indicate the limiting amount of phospholipids rather than the actual TF activity.[18] Second, it has also been shown that FVIIa/phospholipids significantly activate FX,[19–21] especially following long incubation. Hence, the generation of FXa by FVIIa and phospholipids in the absence of TF is also registered as TF activity. Third, auto-activation of FX by FXa[22] may also influence the results independent of TF activity.

For many years, Østerud et al. has used an in-house TF activity assay using vitamin K-dependent coagulation factors (i.e., Ba-citrate eluate being a source for FX, prothrombin, and FVII that is partially activated) in combination with activated bovine FV (FVa), phospholipids, and $CaCl_2$. Following short incubations of 3–4 min, TF activity was quantified using a chromogenic substrate to thrombin.[23] Although highly sensitive and simple to perform, the assay requires standardization to improve the availability for widespread use. Hence, the aim of the present study was to design a sensitive and specific TF activity assay, based on our in-house assay, using commercially available coagulation factors. We show that the assay is highly sensitive and reliable in measuring TF activity in EVs collected from cell culture supernatant, as well as on the surface of live cells or intracellularly. Furthermore, our assay allows the measurement of TF activity in EVs isolated from plasma of healthy volunteers who received low dose lipopolysaccharide (LPS; 2 ng/kg bodyweight).[24]

Essentials

- Non-specific signal may be recorded as tissue factor (TF) activity in TF activity assays.
- Investigation of TF activity requires an assay with high specificity and sensitivity.
- Here, we optimized and tested the performance of a rapid and highly accurate TF activity assay.
- The assay is used to study TF activity in extracellular vesicles, cells, and clinical samples.

2 | MATERIAL AND METHODS

2.1 | Materials

2.1.1 | Reagents

- Human Factor II (Enzyme Research Laboratories, cat. no. HP1002)
- Human Factor X (Enzyme Research Laboratories, cat. no. HFX1010)
- Human Factor VII_a (Enzyme Research Laboratories, cat. no. $HFVII_a$)
- Human Factor VII (Enzyme Research Laboratories, cat. no. HFVII 1007)
- Human Factor VIIa Inactivated (Enzyme Research Laboratories, cat. no. HFVIIai)
- Bovine Factor (BV) V/V_a (Enzyme Research Laboratories, cat. no. BFV/V_a)
- Bovine FXa (Enzyme Research Laboratories, cat. no. BFX_a)
- Dade Innovin (Siemens Healthcare, cat. no. B4212-40)
- Thrombin chromogenic substrate Spectrozyme TH (BioMedica Diagnostics, cat. no. 238L)
- Pefachrome® FXa 8595 (a chromogenic substrate for FXa, 5-Diagnostics AG, cat. no. 085–27)
- UPTT reagent, a standardized rabbit brain cephalin source (Bio/Data Corporation, cat. no. 105997)
- PH 4.0 buffer (Certipur®, cat. no. 1.09435.1000)

- Hepes (Merck/Sigma-Aldrich, cat. no. H3537)
- Sodium chloride (NaCl, VWR Chemicals BDH, cat. no. 7647–14–5)
- Recombinant tumor necrosis factor alpha (TNFα; Merck/Sigma Aldrich cat. no. GF314)
- LPS (Merck/Sigma Aldrich cat. no. O111:B4)
- Ca^{2+}/Mg^{2+}-free Dulbecco's phosphate-buffered saline (DPBS) (Merck/Sigma Aldrich, cat. no. D8537-500 mL)
- Anti-TF-antibody conjugated to PE (BioLegend, cat. no. 365204)
- PE Mouse IgG1, κ isotype control (BioLegend, cat. no. 400112)
- BD Vacutainers (BD Bioscience cat. no. 363048)
- Lymphoprep (Axis Shield cat. no. 1116508)

2.1.2 | Cells

- HAP1 and HAP1-F3KO (Horizon Discovery Ltd.)
- Pooled human umbilical vein endothelial cells (HUVECs; Thermo Fisher Scientific, cat. no. C0155C)
- Iscove's Modified Dulbecco's Medium (IMDM; Thermo Fisher Scientific, cat. no. 31980048)
- Complete endothelial cell growth medium (Cell Applications, cat. no. 211–500)

2.1.3 | Instruments

- Microplate reader, MultiScan FC (Thermo Fisher Scientific)
- 96-well plate (flat bottom, MaxiSorp), Nunc (Thermo Fisher Scientific)
- NanoSight NS300, Malvern Instruments Worcestershire
- Flow cytometer, CytoFLEX, Beckman Coulter Indianapolis

2.2 | TF activity assay

To determine TF activity, 15 μL of EV suspensions in reaction buffer (20 mM HEPES/150 mM NaCl) were incubated with anti-human TF monoclonal blocking antibody (HTF-1) at a concentration of 59 μg/mL, or control IgG for 10 min at room temperature (RT) in 96-well plate. Next, 60 μL of reaction mix containing HFVIIa, HFX, HFII, BFV/Va, and UPPT reagent was added at final concentrations of 34 pM, 18.8 nM, 130 nM, 4.8 μg/mL, and 1/875-dilution, respectively. The performance of HFVII and HFVIIa in the TF assay was compared (see Figure 3); in this set of experiments HFVII was used at 340 pM in the reaction mix, while HFVIIa was used at 340 pM or 34 pM. Plates were pre-warmed in water bath at 37°C for 5 min, and 15 μL of 50 mM $CaCl_2$ in reaction buffer (20 mM HEPES/150 mM NaCl) were added to initiate the reaction. Following reaction of 4 min at 37°C, 30 μL of chromogenic substrate of thrombin (2.5 mM) was added to the mixture and the reaction was allowed for 5 min at 37°C before the reaction was stopped by addition of 50 μL of pH 4.0 buffer. Optical density (OD) was determined immediately using a plate reader at 405 nm wavelength. TF activity was expressed in fM of TF equivalent by comparison to a standard curve generated using Dade Innovin (stock concentration 6 nM). Each sample was analyzed in duplicates, with and without TF blocking antibody.

To test TF activity of cell surface, cells were cultured in a 96-well flat bottom plate. Cells were washed twice with the pre-warmed reaction buffer, incubated for 10 min at RT in 15 μL buffer containing anti-TF antibody or control IgG, and the reaction was initiated as described above. After 4 min 30 μL of chromogenic substrate for thrombin was added. Following 5 min at 37°C, 110 μL of the reaction mixture was transferred to a new 96-well plate containing 50 μL of pH 4.0 buffer per well and OD was determined using plate reader at 405 nm. Cell integrity was confirmed by microscopic examination following the short incubation in reaction buffer/mix. To assess intracellular TF activity, cells were washed three times in reaction buffer and the plates were frozen at −60°C overnight. Cells were thawed in a water bath at 37°C for 2 min, placed on ice, homogenized, and transferred to a new plate, 15 μL per well. The assay was run as described above.

In the experiments in which FVIIa inhibitor (FVIIai) was used, 15 μL of EV suspensions in reaction buffer were pre-incubated with FVIIai at a concentration of 0.12 μg/mL for 10 min at room temperature in the 96-well plate.

2.3 | Autoactivation of FX

FXa at a concentration range from 7.5–0.06 nM in a 2-fold serial dilution was incubated with human FX (150 nM) in presence of phospholipids (UPTT reagent, 125x diluted) and calcium chloride (5 mM) in 20 mM HEPES/150 mM NaCl buffer for 30 min at 37°C, in a final reaction volume of 125 μL. Next, 25 μL of FXa-specific chromogenic substrate (4 mM) was added and the reaction mixture was incubated for additional 15 min at 37°C. OD was determined at 405 nm. To generate standard curve for FXa activity, FXa was used at 7.5–0.06 nM, in a 2-fold serial dilution.

2.4 | FXa generation assay

FVIIa and FX at 4 and 100 nM, respectively, were incubated in presence or absence of phospholipids (UPTT reagent, diluted 125x) in 20 mM Hepes/150 mM NaCl-buffer. The reaction was started by $CaCl_2$ (5mM) and was carried for 2 h at 37°C. Next, FXa-specific chromogenic substrate (4 mM) was added for 15 min and OD was determined at 405 nm. OD values were translated to FXa activity using a standard curve of FXa activity (as described under "autoactivation of FX").

2.5 | Cell culture

Haploid human cell line HAP1 and its derivative TF-knock out cell line (KO F3) were cultured in IMDM supplemented with 10% fetal

calf serum (FCS). Primary HUVECs were cultured in complete endothelial cell growth medium and used up to passage 5. All cells were cultured at 37°C and 5% CO_2. TF surface expression (antigen) was confirmed by flow cytometry.

2.6 | Sample preparation

Blood of healthy donors with written consent was drawn from an antecubital vein into blood collection tubes containing sodium citrate. Platelet-free plasma (PFP) was generated by two consecutive centrifugations at 2500 g for 15 min. EV-depleted plasma (EVDP) was generated from PFP by centrifugation at 100 000 g for 60 min at 4°C. Plasma was stored at −70°C.

Peripheral blood mononuclear cells (PBMCs) were isolated using density gradient (Lymphoprep), and monocytes were further purified by incubating PBMCs in T75 cell culture flasks for 15 min. Monocytes were stimulated with LPS (5 ng/mL) in RPMI medium supplemented with 10% of 0.1 μm pre-filtered FCS for 4 h. EVs were purified from culture supernatants as described in the next section for cell-culture supernatants. TF surface expression on monocytes was confirmed by flow cytometry.

2.7 | Purification of extracellular vesicles

Cell-culture supernatants were collected following 24-h in culture of HAP1-TF+cells that constitutively express TF and from HAP1 *F3*-knock-out (KO) cells, which do not express TF.[25] Supernatants were spun for 5 min at 300 g to remove cells and then centrifuged at 2500 g for 10 min at 4°C to remove cell debris. EVs were precipitated from the supernatant following centrifugation at 20 000 g for 30 min at 4°C. EV pellets were then washed in 2 mL reaction buffer and centrifuged at 20 000 g for 30 min at 4°C. Last, EV pellets were re-suspended in 25 μL of EVDP and kept at −70°C until used.

To recover EVs from EVDP, samples were thawed at 37°C in a water bath, diluted 20x vol/vol in cold reaction buffer, and centrifuged at 20 000 g for 30 min at 4°C. The EV pellet was resuspended in reaction buffer and assayed immediately. Isolated EVs were enumerated by flow cytometry using fluorescein isothiocyanate (FITC)-lactadherin staining and NTA.

2.8 | Surface expression of tissue factor by flow cytometry

Surface expression of TF on HAP1-TF+HAP1-TF/KO was determined using flow cytometry. Cells were detached from cell culture plate using TriplE, washed with DPBS, and stained with anti-TF-PE or isotype control for 20 min on ice. Next, cells were washed and analyzed on CytoFLEX. Data analysis was performed using CytExpert 2.0 (Beckman Coulter).

Extracellular vesicles re-suspended in pre-filtered DPBS (100 kDa) were stained with FITC-lactadherin for specific detection of phosphatidylserine for 20 min on ice. EVs were then washed with pre-filtered DPBS and spun at 20 000 g for 30 min at 4°C. EV pellets were re-suspended in 250 μL pre-filtered DPBS analyzed on Cytoflex at the lowest possible rate, 10 μL/min. Data analysis was performed using CytExpert 2.0.

2.9 | NTA

The concentration and size distribution of EVs were determined using NanoSight NS300 (Malvern Instruments Ltd.) equipped with a 488 nm blue laser and a CMOS camera. Samples were thawed at 37°C water and diluted in pre-filtered DPBS (10 kDa) just before the analysis. Samples were captured at ambient temperature with automatic temperature monitoring. Three separate dilutions of the samples were used for analysis. Each dilution was captured five times for 60 s (camera level 15), and the sample was refreshed between captures. The gasket was cleaned between each sample. The nine resulting videos were analyzed with NTA software version 3.3 (detection threshold 5). Mean values for concentration and size distribution were calculated.

2.10 | The human endotoxemia model

The human endotoxemia model is an established and approved model of a TF-driven, self-limiting, acute inflammatory reaction in healthy human volunteers, as previously described.[24,26] Administration of LPS (2 ng/kg bodyweight, US Standard Reference Endotoxin *E. coli*, CC-RE) induces release of pro-inflammatory cytokines accompanied by transient activation of the coagulation system, fibrinolysis, and of the endothelium.[24]

Twenty healthy volunteers were randomized to receive a bolus infusion of LPS (2 ng/kg bodyweight, 16 subjects) or placebo (4 subjects). Blood was drawn at baseline (1 h before infusion); 0 h (at the time of infusion); and 1 h, 2 h, 4 h, 6 h, and 24 h following administration of LPS, citrated plasma prepared by centrifugation (2000 g for 15 min at 4°C), aliquoted, and stored at −70°C. Plasma EVs were purified as described in Section 2.7 from 400 μL of PFP and tested for TF activity. A written informed consent was obtained from all healthy volunteers who were included in this study.

2.11 | Statistical analysis

Statistical analysis was performed using GraphPad Prism, version 6. TF activity was compared in LPS-treated individuals in controls using a two-tailed nonparametric *t* test. *P*-value <.05 was considered significant. The tests used and magnitudes for *P*-value are indicated in each figure legend.

3 | RESULTS

3.1 | TF-independent FX activation

Commonly used assays for TF activity determine the activity as a function of the measured concentration of FXa in a test sample following rather long incubation times.[17,25] This may account for TF-independent generation of FXa, which would be erroneously measured as TF activity. We first set out to examine *in vitro* conditions which lead to generation of a significant amount of FXa in the absence of TF. As shown in Figure 1A, incubation of FVIIa and FX together with phospholipids (PLs) and Ca^{+2}, in the absence of TF, resulted in generation of FXa. The reaction was largely dependent on the presence of PLs. Autoactivation of FX may also contribute to the generation of FXa in a TF-independent fashion. To assess this possibility FX at concentration of 55 nM was incubated with increased concentration of FXa, and FXa activity was measured using FXa chromogenic substrate. The activity of FXa was increased approximately 2-fold with increasing amount of FXa added to FX following incubation of 30 min (Figure 1B), thus underscoring the possibility of autoactivation of FX.

3.2 | Rapid and specific detection of TF activity

A rapid and highly specific measurement of TF activity can be performed using a two-stage amidolytic assay. The assay is based on the ability of TF-FVII/FVIIa complex to accelerate FX activation (i.e., FXa) followed by conversion of prothrombin to thrombin by FXa in the presence of FV/Va and PLs. The generation of thrombin is proportional to the activity of TF and can therefore be expressed as TF activity.[23] To standardize the original assay we used rhTF and commercially available reagents (see Material and Methods section). As shown in Figure 2A,B, TF activity ≥16 fM was detected following 4 min of incubation. Of note, thrombin generation independent of TF appeared with incubation times exceeding 10 min and was steadily increasing. Thus, reflecting the generation of FXa that is not dependent on TF (no-rhTF, black bars, Figure 2A). To accurately determine a suitable reaction time, increased concentrations of rhTF were used and TF activity was determined at 3, 4, 5, and 6 min. Due to the assay specificity, linearity, and range (15–1000 fM), 4 min incubation time was selected for optimal detection of TF activity. An improved resolution (sensitivity), which allows the detection of lower TF activity, could be achieved by prolonging incubation time to 5 or 6 min (Figure 2B).

Thereafter we examined whether our assay could be used to specifically detect and quantify TF activity on EVs. EVs were isolated from cell culture supernatants of HAP1 cells, which constitutively express TF (hereafter EV-HAP1-TF+). EVs derived from HAP1 *F3*- KO cell line, which do not express TF, were used as negative control (EV-HAP1-TF/KO). TF expression or absence on the surface of HAP1-TF+ and HAP1-TF/KO cells, respectively, was confirmed using flow cytometry (Figure S1 in supporting information). High TF activity was detected in EV-HAP1-TF+ following 4 min reaction time. The activity was blocked using TF-specific monoclonal antibody HTF-1, thus confirming the specificity of the assay (Figure 2C). Higher TF activity was measured following longer incubation times (11 and 20 min), but at the expense of higher background levels. Importantly, TF activity was not detected in EVs isolated from HAP1-TF/KO cells.

3.3 | Usage of FVII and FVIIa in the assay

As FVII can be activated by newly generated FXa in the presence of PLs,[18] we wanted to compare the effects of FVII and FVIIa on the detection of TF activity in our assay. As depicted in Figure 3A, FVIIa

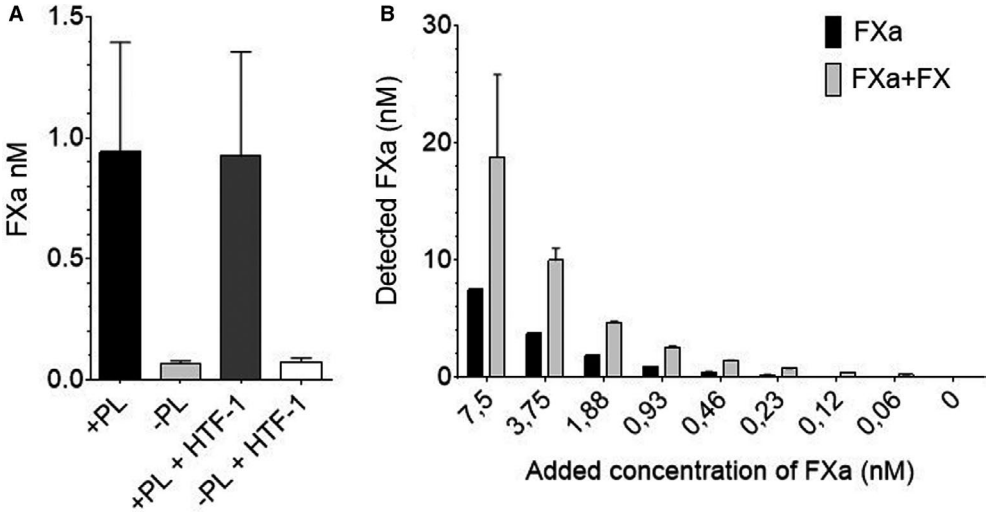

FIGURE 1 Tissue factor-independent activation of coagulation factor X *in vitro*. (A) Activation of factor X (FX) following 2 h incubation of FX in the presence of factor VII (FVII)a, phospholipids, and calcium at 37°C. (B) Autoactivation of FX in the presence of FXa, phospholipids and calcium. FX (55 nM) was incubated with increasing concentrations of FX (0 –7.5 nM) at 37°C for 30 min. FXa activity was measured using chromogenic substrate for FXa

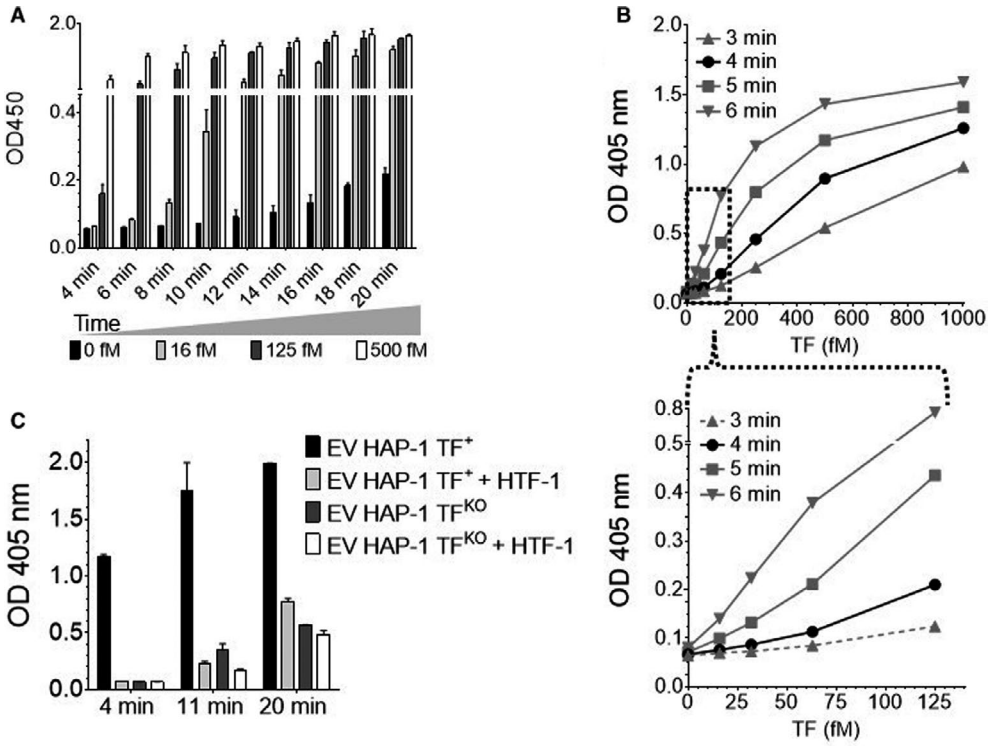

FIGURE 2 Kinetics of tissue factor (TF) activity assay and non-specific activity. (A) Detection of hrTF at 16 fM and 125 fM was assessed following 4–20 min. Non-specific activity was increases with time, TF 0 fM; black bars. (B) Activity of hrTF at 16–1000 fM was assessed using reaction time of 3, 4, 5, and 6 min. Higher resolution of the assay at low concentrations TF is achieved by prolonging the reaction (B, lower panel). (C) TF activity of EVs from HAP1-TF+ and HAP1-TF/KO following reaction time of 4, 11, and 20 min. Samples were pre-incubated with the blocking anti-TF antibody HTF-1 or IgG control. Background levels of registered TF activity is increasing with prolonged reaction time

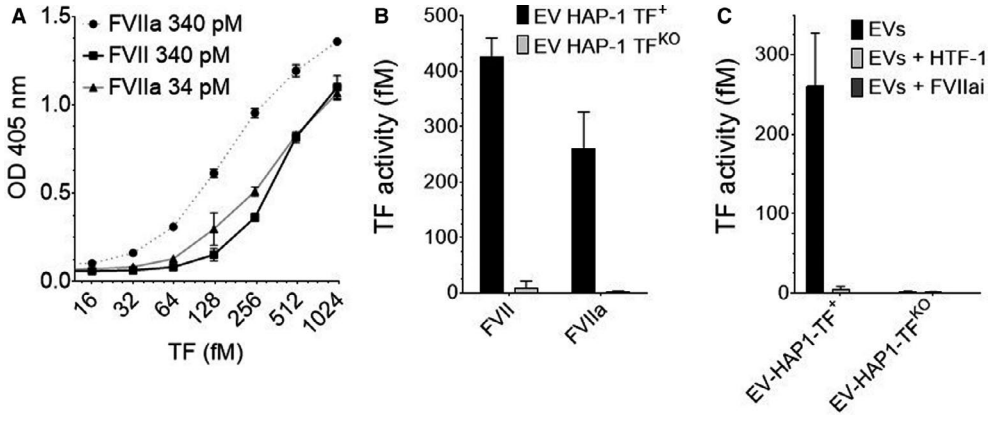

FIGURE 3 Assessment of the performance of factor VII (FVII) and FVIIa in the tissue factor (TF) activity assay. (A) Standard curves of elevated concentrations of hrTF with either FVII (340 pM), or FVIIa at 340 pM and 34 pM. When used at 34 pM, FVIIa showed a comparable performance to FVII used at 340 pM, yet with an increased resolution at lower concentration of hrTF. (B) Measurements of TF activity in EV-HAP1-TF+ and EV-HAP1-TF/KO with FVII (340 pM) or FVIIa (34 pM). (C) Blocking of TF activity measured in EV using anti-TF antibody HTF-1 or FVIIai. FVIIa was used in this set of experiments

provided rapid generation of thrombin under the conditions set for our TF activity assay. To achieve similar performance of FVII and FVIIa, FVII (340 pM) was used at a concentration 10 times higher than FVIIa. Compared to FVII, FVIIa showed a steeper and more linear curve at lower concentrations of TF (Figure 3A). At higher TF concentrations, the performance of FVII (340 pM) and FVIIa (34 pM) were similar. However, higher TF activity was measured in EVs from HAP1-TF+ using FVII (340 pM) rather than FVIIa (34 pM; Figure 3B). To further examine the importance of FVII/FVIIa/TF complex in the system, EVs were pre-incubated with FVIIa inhibitor (FVIIai). FVIIai competes with FVIIa and forms a complex with TF, yet lacks enzymatic activity of FVIIa. In this experiment FVIIa was used. As

depicted in Figure 3C, FVIIai completely blocked thrombin generation, further supporting the specificity of the assay and the central role of FVII/FVIIa/TF complex in the TF activity assay.

3.4 | Measurement of TF activity on cell-derived extracellular vesicles and cell surface

To assess the ability of the assay to accurately quantify TF activity when working with EVs, TF activity was determined on serial dilutions of EV-HAP1-TF+. As shown in Figure 4A, the signal from EV-HAP1-TF+ was proportional to the dilution of the samples.

Next, we examined the possibility of detecting TF activity on the surface of live cells. To keep cells alive for potential downstream analyses the assay was moderately modified: The reaction mixture of different factors, which was not toxic to cells, was added directly to the original culture plate. Next, 110 uL of the reaction mixture was transferred to a new plate containing quenching buffer (pH 4.0). HAP1-TF+ and their HAP1-TF/KO counterpart cells were tested for surface TF activity. As shown in Figure 4B, surface activity of TF was detected solely on HAP1-TF and not on HAP-1 KO.

3.5 | Measurement of cell surface and intracellular TF activity in primary cells

Tissue factor activity was further examined on the cell surface of primary HUVECs and in the cell lysates. Stimulation of HUVECs with LPS or TNFα for 4 h induced moderate, yet detectable, levels of surface TF activity (Figure 5A). To determine the total TF activity of HUVECs, that is, surface and intracellular activity, LPS- and TNFα-stimulated cells were frozen, thawed, and homogenized following surface TF activity measurement. Interestingly, the total TF activity of HUVECs following activation with TNFα was >30-fold higher than the surface activity (Figure 5A).

Surface and total TF activity were also tested in primary human monocytes. High levels of TF activity were found on monocyte surface following 4 h stimulation with LPS (Figure 5B). Similar TF activity was measured following homogenization, suggesting that the major proportion of TF in monocytes is transferred to the cellular surface upon cellular activation and translation of TF.

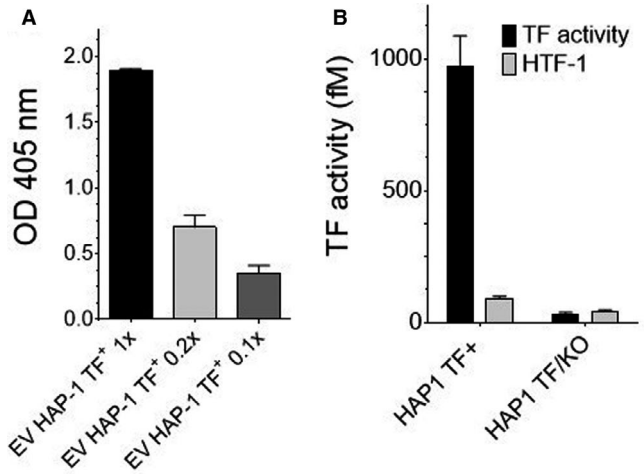

FIGURE 4 Measurements of tissue factor (TF) activity in extracellular vesicles (EVs) from on cell surface. (A) TF activity levels measured in different concentrations of EV-HAP1-TF+ (1X, 1/5X, and 1/10X) generate proportional signal. (B) TF activity was measured on the surface of HAP1-TF+ and HAP1-TF/OK cells. Cultured cells were pre-incubated with anti-TF antibody HTF-1 or IgG control prior to the TF activity assay

3.6 | TF activity in EVs isolated from human individuals who received LPS and in controls

To mimic an acute inflammatory response, healthy volunteers were treated with low levels of LPS, 2 ng/kg bodyweight[24] and plasma samples were collected before and at different time intervals after the LPS treatment. The study includes 20 individuals: 16 who were treated with LPS and 4 subjects who were treated with placebo, and thus used as controls.[24] EVs were isolated from plasma collected before the study (−1 h); at the time of LPS administration (0 h); and 1 h, 2 h, 4 h, 6 h, and 24 h following LPS treatment. TF activity in isolated EVs was detected after 2 h, peaked at 4 h, and maintained at rather high levels 6 h after LPS treatment (Figure 6). Trace amounts of TF activity were also found in some individuals following 24 h (Figure 6 and Figure S2 in supporting information). No TF activity was found in placebo controls.

3.7 | Variability and reproducibility of the TF activity assay

Finally, we have evaluated the coefficient of variation (CV) of the assay using lysates of HAP1-TF+ cells. The intra-assay (replicate assays of the same sample) and inter-assay (day-to-day) variation were rather low, 9.1% and 11%, respectively.

4 | DISCUSSION

Tissue factor is the primary activator of the coagulation cascade; it plays a critical role in hemostasis and is essential for life.[27,28] Aberrant expression of TF in the vascular system has been associated with pathological thrombotic processes.[29–31] However, an accurate and reliable measurement of its activity has proven difficult, and for many years has been performed using time-consuming assays with limited specificity and sensitivity.[14,15,27] These limitations impede the study of TF involvement in pathological conditions, as well as the use of its activity as a biomarker for prediction and/or diagnosis of thrombosis. Accordingly, the main goal of this study was to validate a sensitive and accurate TF activity assay, using commercial reagents that can easily be performed in research and clinical laboratories.

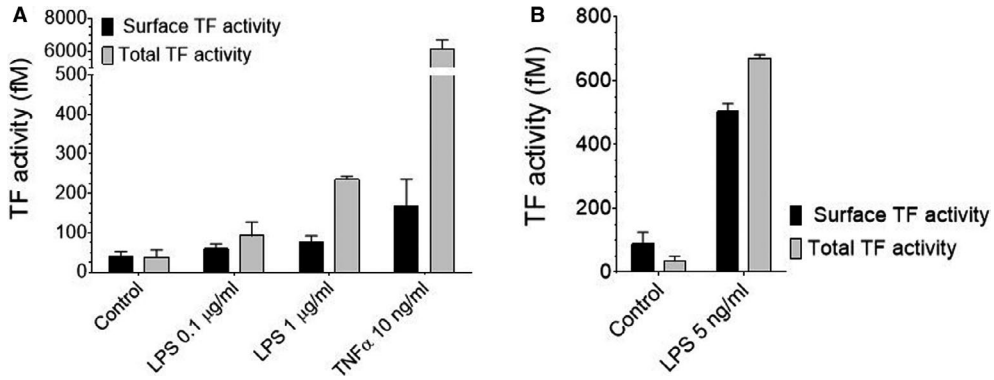

FIGURE 5 Measurements of surface and total tissue factor (TF) activity in primary cells. (A) Human umbilical vein endothelial cells (HUVECs) were *in vitro* stimulated with lipopolysaccharide (LPS) and tumor necrosis factor alpha (TNFα) for 4 h in culture, and (B) monocytes were stimulated with LPS and TF activity was measured on cell surface. Next, cells were washed and frozen overnight at −60°C. Cells were homogenized and total TF activity was measured in the cell lysates

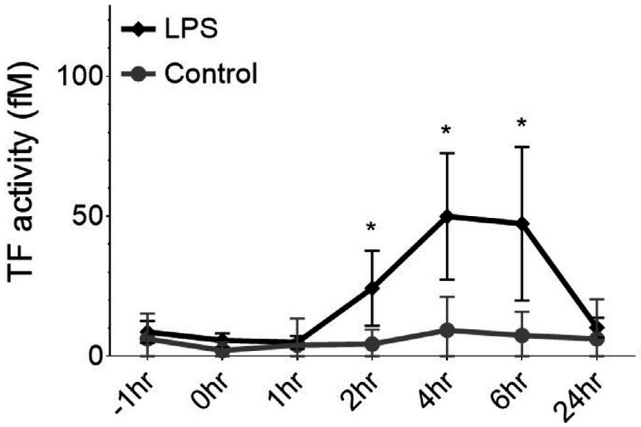

FIGURE 6 Tissue factor (TF) activity is increased in human endotoxemia model. Extracellular vesicles (EVs) were isolated from plasma of healthy volunteers who received low concentration of lipopolysaccharide (LPS; $n = 16$) or received a placebo ($n = 4$). Samples were collected at baseline (–1 h); at the time of infusion (0 h); and 1 h, 2 h, 4 h, 6 h, and 24 h following treatment. TF activity was measured in EVs and presented as the mean with 95% confidence interval of the treated individuals and placebo controls. The individual responses are shown in Figure S2. $^*P < .05$

Our new TF activity assay is sensitive, specific, rapid, easy to perform, and can be used to accurately measure TF activity in clinical samples and *in vitro* experimental systems on live cells. However, one limitation of our TF activity assay may be its inability to detect soluble TF that is not bound to microvesicles.[32] Using optimal concentrations of commercially available coagulation factors (FVII/FVIIa, FX, FV, and prothrombin), TF activity is rapidly measured in a two-stage thrombin generation assay, quantifying the amount of thrombin generated by the use of a chromogenic substrate. The multiple components that are included in the assay should perform optimally, hence controlled periodically. The superior specificity of the assay is obtained by limiting the incubation times (4–6 min) to avoid non-specific activation of FX by FVIIa and phospholipids, which may cause substantial generation of FXa while working with such mixtures, even in the absence of TF.[18,33] As previously described and further demonstrated in this work, long incubation times of 1–2 h may contribute to significant formation of FXa in the absence or in the presence of trace amounts of TF.

Auto-activation of FX by FXa is also independent of TF activity, yet can be erroneously measured as such, although some degree of autoactivation of FX was acknowledged already in 1974.[22,34,35] However, this phenomenon attracted little attention as it may not play an important role in hemostasis/thrombosis, because only a minor amount of FXa is generated. As shown here, autoactivation of FX is increased with increasing amounts of FXa in a test tube, thereby affecting the registered quantity of FXa that is detected in a test tube. Together with the activation of FX by FVIIa and phospholipids, the two may partially explain reports of high TF activity associated with activated platelets.[36–38]

The activation of FX independent of TF highlights the importance of eliminating non-specific TF activity in the assay. Recent recommendations for standardization of TF activity assays underlined the importance of including TF blocking antibody (clone HTF-1) and/or an inhibitor for FVIIa (FVIIai) active site in order to identify and exclude detection of TF-independent activity.[39] Thrombin generation that is independent of TF activity is detected in our assay when a prolonged reaction time is permitted. The assay is therefore performed rapidly (4–6 min), with minimal detection of background levels. In addition, the use of anti-TF blocking antibody is routinely implemented in the assay and background levels are subtracted. TF-independent thrombin generation in the presence of FVII/FVIIa, FX, and prothrombin is most likely mediated in our assay by autoactivation of FX (as soon as a trace amount of FXa is generated in the reaction mix) and FVIIa and possible by trace amounts of FXa in the FX reagent.

Both native FVII and FVIIa have been used in different TF activity assays,[14,25,40,41] and their performance has been tested and compared in our assay as well. In our assay the use of FVIIa (34 pM) compared to FVII (340 pM), resulted in a higher resolution at very low levels of TF. In contrast, at high concentration of TF, native FVII, which is activated by FXa and PS, mediated higher amounts of thrombin. This can be explained by a feedback activation of FVII as soon as FXa is formed. Because FVII is used at higher concentration

than FVIIa, its feedback activation rapidly results in higher FVIIa than originally desired. This mechanism can also explain the advantage of using FVII rather than FVIIa in a recently reposted version of the Chapel Hill TF activity assay, in which long incubation is permitted and thus also the generation of FVIIa in the reaction mix.[25]

We demonstrated that our assay had the ability to detect TF activity with high specificity in EVs isolated from culture supernatants and plasma samples from healthy subjects who received low doses of LPS (i.e., human endotoxemia model). TF activity was detected in EVs derived from HAP1-TF+ cells but not from EVs HAP1-TF/KO cells. The specificity of TF activity detected in EVs was demonstrated using either anti-TF blocking antibody or by inhibiting the active site of FVIIa using FVIIai. The detected TF activity using these inhibitors was at the background level. The accuracy of the assay was demonstrated by measuring TF activity for serial diluted EVs, which provided proportional signals and low CVs.

The high sensitivity and specificity of our assay allowed us to reliably detect TF activity in EVs isolated from plasma of healthy volunteers who were treated with low dosages of LPS (2 ng/kg bodyweight).[24] Treatment with low dosages of LPS in these subjects induced TNFα and IL-6 production and release, which peaked in plasma within 2 h, whereas markers of endothelial cell activation (E-selectin and von Willebrand factor) and coagulation activation (prothrombin fragment 1+2 and thrombin–antithrombin [TAT] complexes) peaked 4 h after LPS administration.[24,26,42] Accordingly, the TF activity of EVs isolated from plasma peaked in parallel with coagulation activation (prothrombin fragment 1+2 and TAT complexes) *in vivo* but 2 h after the peak of IL-6 and TNFα were detected in samples from the same study.[24] These findings suggest that low-dose treatment with LPS induced monocyte activation and TF expression, which may be further amplified by a release of inflammatory cytokines. These trigger the release of TF-positive EVs, which subsequently activate coagulations *in vivo*.

Another application of the assay was to quantify surface TF activity on intact cells in cell culture (as similarly described by Egorina et al.[43] and Bancsi et al.[44]). This was accomplished by allowing live cells to react with the test reagents within the original culture plate, whereas the reaction was stopped and the OD of chromogenic substrate measured in a new analysis plate. HAP1-TF+ cells possessed high amounts of TF activity that could be abolished by anti-TF antibodies, whereas HAP1-F3/KO cells lacked any TF activity. In addition, we show that our assay could be used to measure TF in primary cells. Surface TF activity was detected on the surface of HUVECs and monocytes following stimulation. Importantly, measurement of TF activity on cell surface using our assay allows for performance of additional analyses as the cells are kept in culture. Total TF activity was measured following a freezing and thawing cycle, which facilitates physical homogenization of the cells. Interestingly, while monocytes show rather comparable surface and total TF activity, HUVECs showed higher total TF activity compared to the activity on their surface, in particular following TNFα stimulation. The relatively high total TF activity of HUVECs compared to monocytes may reflect a difference in permeability of HUVECs, being adherent cells, and monocytes that were in suspension. Our results show that the TF activity assay is applicable for testing and quantifying TF activity at the cell surface and cell lysates in cell lines and primary cells.

In this work we describe a rapid, and easy to perform TF activity assay, which is highly sensitive and specific. The assay is versatile and can be easily adapted to measure TF activity in EVs from cell culture, plasma, on the surface of live cells, or intracellularly. We demonstrate its usage in different applications and showed that the assay can be used to quantify TF activity of EVs in plasma. We believe our assay can be used to test clinical samples and will facilitate future research of TF activity in pathological conditions.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest.

AUTHOR CONTRIBUTIONS

B. Østerud designed and participated in the laboratory analysis, analyzed the data, and drafted and revised the manuscript. N. Latysheva designed and participated in the laboratory analysis, performed the laboratory analysis, analyzed the data, and drafted and revised the manuscript. C. Schoergenhofer and B. Jilma collected plasma samples from endotoxemia models and revised the manuscript. .J-B. Hansen and O. Snir designed the study and participated in the writing and revision of the manuscript. All the authors read and approved the final manuscript.

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