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RESEARCH LETTER

An anti–tissue factor pathway inhibitor antibody increases tissue factor activity in extracellular vesicles isolated from human plasma

We and others have developed assays to measure levels of tissue factor (TF) activity in extracellular vesicles (EVs) isolated from human plasma [1–5]. TF present in plasma would be expected to bind its ligand factor (F)VII/VIIa. TF pathway inhibitor (TFPI) is the primary regulator of TF activity. TFPI forms a quaternary complex with the TF/FVIIa/FXa complex via binding to FVIIa and FXa with its first and second Kunitz domains, respectively [6].

Franco et al. [5] recently reported the isolation of EVs from plasma using immunomagnetic separation (IMS) beads coated with CD29 and CD59 antibodies. Interestingly, TF activity of IMS-captured EVs from the human pancreatic cell line BxPC-3 was lower after incubation in plasma compared to 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer, indicating that a soluble component in plasma reduced TF activity of the EVs. Further, they showed that TF activity of IMS-captured EVs was reduced in normal plasma but preserved in TFPI-deficient plasma or in the presence of an anti-TFPI antibody. The authors concluded that TFPI from plasma binds to the IMS beads and inhibits TF activity.

We determined the effect of an anti-TFPI monoclonal antibody 2H8 [7] against the first Kunitz domain on TF activity of EVs isolated from plasma from 4 healthy individuals with or without stimulation of whole blood with bacterial lipopolysaccharide (LPS) (*Escherichia coli* 0111:B4) for 5 hours at 37 °C [8]. EVs were isolated by centrifuging plasma samples at 20,000*g* for 15 minutes at 4 °C, washed, repelleted, and resuspended in buffer [8]. EVs were incubated with 2H8 (15, 30, or 100 μg/mL, final concentration) or immunoglobulin G (IgG) control antibody. A FXa generation assay with an inhibitory anti-TF antibody was used to measure TF activity [8]. 2H8 increased TF activity of EVs isolated from LPS-stimulated blood in a dose-dependent manner compared to a control antibody (Figure 1). As expected, there were differences between individuals. Addition of 2H8 and IgG led to a dose-dependent increase in TF activity in EVs from LPS-stimulated blood (2H8: 15 μg/mL, 0.97 ± 0.08 pg/mL; 30 μg/mL, 1.23 ± 0.17 pg/mL; 100 μg/mL, 3.04 ± 0.52 pg/mL; IgG: 15 μg/mL, 0.65 ± 0.09 pg/mL; 30 μg/mL, 0.64 ± 0.19 pg/mL; 100 μg/mL, 0.97 ± 0.36 pg/mL; mean ± SD). The average fold increase in TF activity in EVs isolated from LPS-stimulated samples and incubated with 15 μg/mL, 30 μg/mL, and 100 μg/mL of 2H8 compared to IgG was 1.6-fold, 1.9-fold, and 3.6-fold, respectively. This result indicated that TFPI was inhibiting TF activity of the EVs.

Next, we determined if we could detect TFPI within EV preparations by Western blotting. EVs were isolated from 400 μL of plasma with and without LPS stimulation, as described above. Samples were resuspended in sample buffer with β-mercaptoethanol (2.5% final concentration) and subjected to sodium dodecyl-sulfate polyacrylamide gel electrophoresis. Proteins were transferred to a polyvinylidene difluoride membrane and placed in a 5% nonfat dry milk blocking buffer. The membrane was incubated with a rabbit polyclonal anti-TFPI antibody (1:1000, final concentration 3 μg/mL) [7] overnight at 4 °C, washed, and then incubated with an anti-rabbit IgG conjugated to horseradish peroxidase. Membranes were developed using chemiluminescence. If TFPI was binding to the TF/FVIIa complex on EVs, we would expect to observe higher levels of TFPI in EVs isolated from LPS-stimulated blood compared to EVs isolated from unstimulated blood. However, a similar amount of TFPI was present in EVs isolated from plasma with or without LPS stimulation (Figure 2). This finding suggests that the TFPI in the isolated EV preparations is residual plasma TFPI that copurifies with the EVs rather than TFPI directly binding to the EVs. We propose that FXa generated in the TF activity assay allows rapid binding of TFPI to the TF/FVIIa complex, and this inhibits its activity.

In conclusion, TFPI is present in EV preparations isolated by either centrifugation or IMS beads and inhibits TF activity. The sensitivity of detection of TF activity of EVs isolated from plasma can be enhanced by performing the FXa generation assay in the presence of an anti-TFPI antibody.

FUNDING

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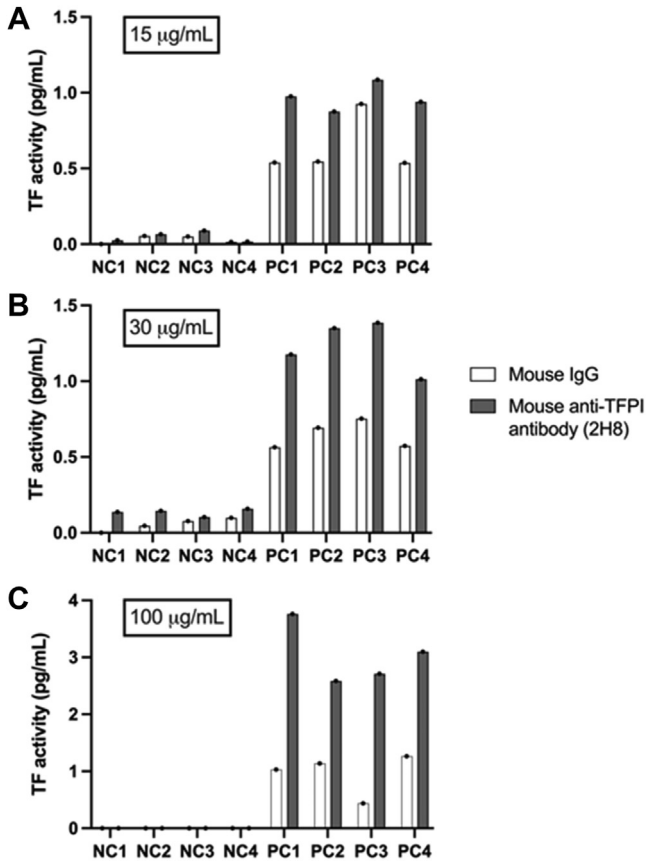

FIGURE 1 Analysis of the effect of an anti–tissue factor pathway inhibitor (TFPI) antibody on tissue factor (TF) activity of extracellular vesicles isolated from human plasma. Whole blood was collected from 4 healthy individuals and divided into 2 portions. A negative control (NC) plasma was prepared by centrifuging the whole blood immediately. A positive control (PC) plasma was prepared by centrifuging whole blood after stimulation with bacterial lipopolysaccharide for 5 hours at 37 °C. Extracellular vesicles were isolated from plasma by centrifugation, washed, repelleted, and incubated with either 2H8 or a control immunoglobulin G (IgG), and then TF activity was measured. The mean fold increase in TF activity in the presence of 15 µg/mL, 30 µg/mL, and 100 µg/mL of the anti-TFPI antibody 2H8 compared to IgG was 1.6 (range, 1.2-1.8), 1.9 (range, 1.7-2.0), and 3.6 (range, 2.3-6.2), respectively.

ETHICS STATEMENT

Ethics statement All healthy donors gave consent for the collection of blood according to a protocol (14-2108) approved by the Institutional Review Board of the University of North Carolina at Chapel Hill.

AUTHOR CONTRIBUTIONS

P.T. and A.T.A.S. conducted experiments, analyzed data and edited the manuscript. A.E.M. edited the manuscript. N.M. designed experiments, interpreted data, wrote the manuscript and edited the manuscript. All the authors have read and approved the final manuscript.

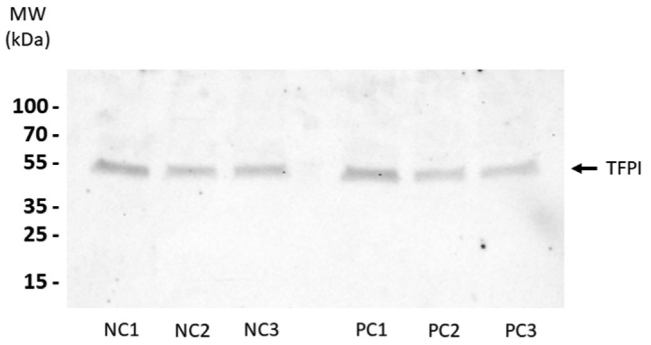

FIGURE 2 Western blot of tissue factor pathway inhibitor (TFPI) in extracellular vesicles. Extracellular vesicles were isolated from negative control (NC) and positive (lipopolysaccharide-stimulated) control (PC) plasmas. Proteins were separated by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and Western blotting was used to detect TFPI using a polyclonal anti-TFPI antibody (1:1000; final concentration, 3 µg/mL). MW, molecular weight.

RELATIONSHIP DISCLOSURE

The authors have no relevant conflicts of interest to disclose.

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