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Microparticles exposing Tissue Factor in human plasma

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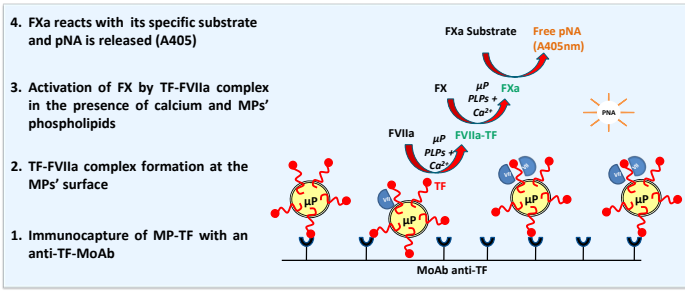
Introduction

- Tissue Factor-exposing Microparticles (MP-TF) are produced in various pathological states including : cancer, atherosclerosis, diabete, acute coronary syndrome, multiple myeloma and sepsis. They can trigger blood coagulation[1].
- MP-TF activity has an **unfavorable prognostic value in Acute Myocardial Infarction** (AMI)[2].
- MP-TF activity may be used as a **biomarker** for evaluating the **risk of disseminated intravascular coagulation** in endotoxemia[3].
- Existing methods for determining MP-TF concentration are flow cytometry, or chromogenic assays. While flow cytometry allows to quantify precisely the number of microparticles, it does not give any information about their procoagulant potential. Homogeneous chromogenic assays (without a specific capture step) do not permit to avoid interferences from other plasma factors or from TF-free microparticles.

Aim

Among microparticles (MP) those exposing Tissue Factor (TF) are of particular interest, for their critical role in the initiation of thrombosis. They may be an useful biomarker to identify an increased risk of thrombosis in various pathologies (e.g. cancer) which contribute to disease complications. We developed the Zymuphen MP-TF method, an ultra sensitive bio-immunoassay, that allows the determination of MP-TF procoagulant activity in human plasma.

Assay principle



Materials and Methods

Plasmas are prepared using a double centrifugation (15 min. at 1500g and 2 min. at 13.000g) at room temperature to eliminate platelets.

Lipopolysaccharide stimulation (LPS): Whole heparinized blood is incubated with LPS (O111:B4 from Sigma) and plasmas are prepared as here above. Control is plasma prepared from the same untreated blood.

Specificity: Either LPS-induced plasmas, or kit controls high and low, have been spiked with an anti-TF polyclonal Ab (HTI) at 2 µg/ml, or 0 µg/ml (controls), and incubated at 37°C for 2 hours before testing for MP-TF.

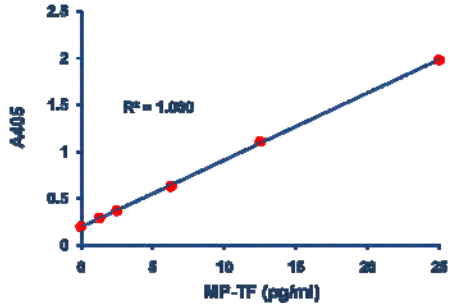
Coated microplate: MoAb specific for extracellular domain of Tissue Factor, that does not interfere with TF activity.

R1: recombinant human Factor VIIa (NovoSeven®), **R2**: highly purified human Factor X, (Hyphen BioMed), **R3**: Factor Xa specific substrate CS 11-(65) (Hyphen-Biomed).

Calibration and controls: Full length recombinant Tissue Factor (1-263) (ADI) has been relipidated with synthetic liposomes (HBM) and lyophilized with stabilizers. MP-TF concentration is expressed as TF antigen equivalent in pg/ml.

Results

CALIBRATION CURVE



REPRODUCIBILITY AND REPEATABILITY

	Inter-assay			Intra-assay		
	N series	Mean (pg/ml)	CV (%)	N replicates	Mean (pg/ml)	CV (%)
CI [13-19 pg/ml]	7	16.3	**6**	12	17.1	**5**
CII [6-9 pg/ml]	7	7.3	**5**	12	7.5	**5**

NORMALS

N	Mean Conc. (pg/ml)	Min (pg/ml)	Max (pg/ml)
19	**0.5** (below detection limit = 1 pg/ml)	0.0	2.0

➡ Increased in pathology: 2.0 to > 50.0 pg/ml (TF Eq.)

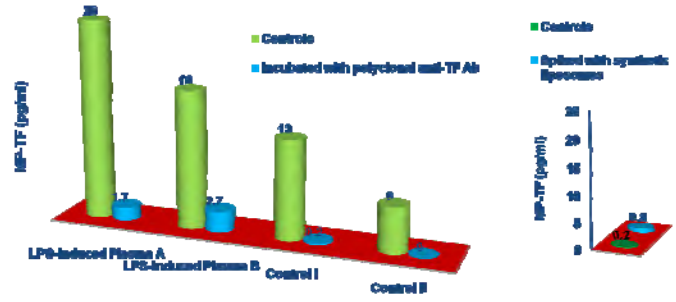
PATHOLOGICALS

N	Mean Conc. (pg/ml)	Median (pg/ml)	Min (pg/ml)	Max (pg/ml)
19	**3.0**	**0.2**	0.0	>25.0

LPS-INDUCED PLASMA

➡ LPS induces an important release of MP-TF in whole blood.

SPECIFICITY



➡ When incubated with an anti-TF polyclonal antibody, all MP-TF activity is blocked.

➡ There is no reactivity with TF-free liposomes (400 x conc. of kit calibrator), with or without truncated TF (1, 219).

Conclusions

➡ Zymuphen MP-TF is a **highly sensitive and reproducible** method for the measurement of MP-TF in plasma, and is an useful tool for assessing the clinical interest of this biomarker in pathology.

➡ Plasmas from **LPS-induced blood release MP-TF which can be measured** using **Zymuphen MP-TF**, in a **TF-specific reaction**.

➡ **Synthetic liposomes**, that mimicks TF-free microparticles, **do not react** with Zymuphen MP-TF.

References

1. Morel et al. **Procoagulant microparticles: disrupting the vascular homeostasis equation?** *Arterioscler Thromb Vasc Biol* 26:2594-2604, 2006.

2. Steppich et al. **Plasma TF activity predicts cardiovascular mortality in patients with acute myocardial infarction**. *Thromb J*, 7:11, 2009.

3. Wang et al. **Levels of microparticle tissue factor activity correlate with coagulation activation in endotoxemic mice**. *J Thromb Haemost*, 7(7):1092-1098, 2009.

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Aim: Among microparticles (MP) those exposing Tissue Factor (TF) are of particular interest, for their critical role in the initiation of thrombosis. They may be a useful biomarker to identify an increased risk of thrombosis in various pathologies (e.g. cancer). We developed the Zymuphen MP-TF method, an ultra sensitive bio-immunoassay that allows the determination of MP-TF procoagulant activity in human plasma.

Method: Plasmas are prepared using a double centrifugation at Room Temperature (low and high speed) to eliminate platelets. MP-TFs are captured by a murine MoAb directed against the extracellular domain of TF, that does not inhibit TF activity. Following a washing step, FVIIa and F X are added into the reaction mixture. TF-FVIIa complexes form and activate F X into F Xa (FXa) in presence of Ca++. FXa generation is dependent on TF and MP's anionic phospholipids concentration. Then a FXa-specific substrate (CS 11(65)) is added, reacts with FXa, releasing pNA, which absorbance is recorded at 405nm. A lyophilized calibrator, containing rec.(h) TF relipidated with synthetic phospholipids, permits the standardization of the assay. Calibration is from 0 to 5 pg/ml of TF equivalent, with a ratio of 0.1 nM PS/ 1 pg TF.

Results: Normals were < 0.2 pg/ml, while 2 pathological plasmas that were found to have high MPs concentration (9.8 and >63 nM PS) with Zymuphen MP Activity, were found at 0.95 pg/ml and > 5 pg/ml using Zymuphen MP-TF. Plasmas from LPS-stimulated whole blood following a 6 hour incubation were significantly higher than baseline (t=0h) with a MP-TF generation ranging from 5 to 22 µg/ml. Recombinant Truncated TF (1-219), when mixed at 100 pg/ml with synthetic saturating phospholipids (870nM PS), was < 0.2 pg/ml demonstrating the specificity for MP-TF.

Conclusion: Zymuphen MP-TF is a highly sensitive and specific method for the measurement of MP-TF in citrated plasma, and is a useful for assessing the clinical interest of this biomarker in pathology.