Contact activation: important to consider when measuring the contribution of tissue factor-bearing microparticles to thrombin generation using phospholipid-containing reagents

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Contact activation – important to consider when measuring the contribution of tissue factor-bearing microparticles to thrombin generation using phospholipid-containing reagents

Running title: Impact of FXII on TF-MP measurements using TG

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Abstract

Background
A commercial MP reagent containing phospholipids is used for Thrombin generation (TG) measurements to estimate the procoagulant activity of microparticles (MPs). Previous reports have shown that contact activation affects TG when TF levels are low, and that addition of phospholipids might augment this effect.

Objectives
To quantify the impact of contact activation on TG in the presence of phospholipids and low/no TF, as is the case using a commercially available MP-reagent.

Methods
Thrombin generation was analyzed using MP- or PRP-reagent in the presence and absence of CTI and anti-TF antibodies, respectively. To quantify the impact of different experimental parameters on contact activation, microparticle-depleted plasma was analyzed in the presence of different concentrations of phospholipids, TF and/or contact activating agents (kaolin).

Results
Even with low-contact activating blood collection tubes, substantial thrombin generation was observed with the MP-reagent, but this was c completely inhibited by CTI addition. Control experiments illustrate that the phospholipids in the reagent plays a major role to enhance TG initiated by FXIIa. Even with the PRP-reagent, suggested to be used to determine the content of phospholipids from MPs, TG was partly dependent on contact activation.

Conclusions

Contact activation plays a major role for TG when using reagents/samples containing phospholipids but little or no tissue factor. This needs to be considered and accounted for in future clinical studies using TG to assess the procoagulant activity of MPs.

Keywords. blood coagulation; cell-derived microparticles; factor XII; thrombin; thromboplastin.

Introduction:

Thrombin generation (TG) has been proposed as a method to estimate the procoagulant activity of microparticles (MPs). TG could be considered an attractive alternative for use in clinical studies, as the method confers several practical advantages, such as the use of frozen samples, ease of use and straightforward interpretation of data. Commercial kits for the assessment of MP contribution to TG are available (Thrombinoscope®, Maastricht, The Netherlands), and it has been proposed that the marketed PRP reagent, with minimal amounts of phospholipids and 1 pM tissue factor (TF), could be used to estimate the contribution of MP phospholipids, while the MP-reagent which is devoid of TF, but with 4 µM phospholipids, could be used to estimate the contribution of TF-bearing MPs.

It has previously been reported that contact activation influence TG at low concentrations of TF [1-5]. The kit insert delivered with the MP reagent therefore proposes to use corn trypsin inhibitor (CTI) to prevent factor XII activation. When CTI should be added is not specified in the insert, however, and due to the impracticalities and costs associated with adding CTI to the blood collection tubes, it is possible that such measures could be omitted in the absence of more conclusive evidence quantifying the effects on analytical precision caused by contact activation when using phospholipid-containing reagents. Furthermore, it is also common to consider to use frozen samples collected primarily for other purposes, which will naturally not have been collected in the presence of CTI. We and others have previously shown that the contribution of contact activation is amplified in the presence of phospholipid membranes [6, 7]. Therefore, this study was designed to measure the impact of contact activation on TG in the presence of phospholipids and low/no TF. In this study we focus on the impact of contact activation on TG when using the abovementioned MP-reagent.

Materials and Methods:

To assess the impact of contact activation on TG using the commercial MP- and PRP reagents, blood was drawn from healthy volunteers and collected in 7.5 mL S-Monovette® tubes (Sarstedt, Nümbrecht, Germany) with 1/10 (v/v) citrate (130 mM), as these tubes were previously

demonstrated to cause minimal pre-analytical contact activation [8]. Plasma was obtained by double centrifugation at 2500×g for 15 minutes within 1 hour of blood collection, as recommended in current guidelines for microparticle detection [9]. Plasma samples were analyzed using the MP-reagent or PRP-reagent, as is or in the presence of the FXII-inhibitor corn trypsin inhibitor (CTI, 100 µg/ml; Haematologic technologies Inc., Essex Junction, VT, USA) or polyclonal rabbit antibodies directed against human tissue factor (TF-Ab, 100 µg/ml, proven to block tissue factor-induced coagulation; American Diagnostica, Stamford, CT, USA).

Results:

As shown in Figure 1, synthetic phospholipids dramatically enhance factor XII-dependent coagulation and thrombin generation in microparticle-depleted platelet-free plasma filtered through a 0.20 µm Minisart® filter (Sartorius Ltd, Surrey, UK). Filtration of plasma through these filters did not in itself cause FXII activation and did not affect subsequent kaolin-induced FXII activation (data not shown). Even without exogenously added factor XII-activator (kaolin), 0.5-2.0 µM synthetic phospholipids was sufficient to produce robust thrombin generation within 15 minutes, and increasing contact activation by kaolin both decreased the lag time and increased the peak of thrombin generation (fig 1, n=5).

Even when using low-contact activating blood collection tubes, substantial thrombin generation was observed with the MP-reagent. This could easily be interpreted as a high level of circulating TF-exposing MPs, especially as the kit insert mentions that a long lag time and little or no thrombin generation should be expected in absence of tissue factor, which could be assumed to be the case in normal donors. However, thrombin generation was completely absent in the presence of 100 µg/ml CTI, while being virtually unaffected by the addition of 100 µg/ml of TF-blocking Ab, indicating that thrombin generation was completely dependent on contact activation under these conditions. When using the PRP-reagent described above, thrombin generation was lower, and a partial dependency on both contact activation and TF was observed, illustrating that contact activation contribute to thrombin generation even under these conditions (fig 2 and table).

Conclusions:

We conclude that contact activation has to be taken into account if these reagents are to be used to estimate the contribution of TF-bearing MPs to TG. One problem is that the extent of contact activation will vary with a number of factors, including the conditions and tubes used for blood

collection [8, 10], and can therefore not be easily estimated or subtracted. As previously mentioned, we fear that the addition of CTI to blood collection tubes may not be considered feasible for larger/multi-center clinical studies, both due to costs and problems with special preparation/manipulation of the blood collection tubes, and impossible to apply when considering analysis of already collected plasma samples. In such cases, an alternative strategy may be to use methods including parallel samples containing an antibody towards TF to clarify the relative contribution of TF [11, 12]. Another approach that has been tested for clinical samples is to isolate the microparticles by centrifugation and add them to CTI-treated normal plasma before performing the thrombin generation assay [13, 14].

With this study, we want to raise the awareness of the large impact of artefactual contact activation on thrombin generation when using reagents containing phospholipids but little or no tissue factor, as exemplified by the MP-reagent. Unless accounted for, this may lead to an overestimation of the contribution of TF to thrombin generation. Therefore, measures to control and reduce the impact of contact activation need to be discussed and actions taken to avoid this when planning clinical studies designed to assess the role and importance of TF-bearing MPs.

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Figures

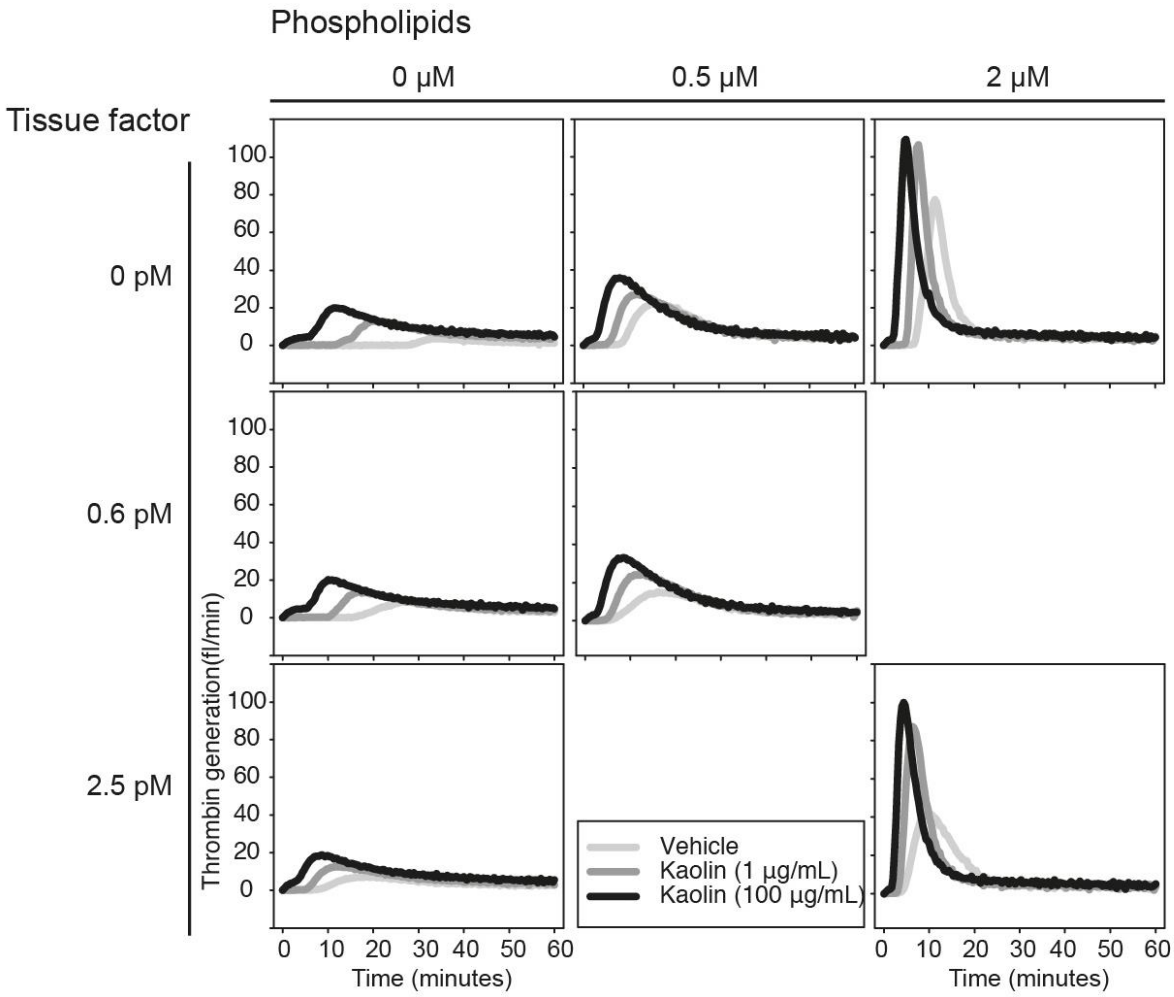
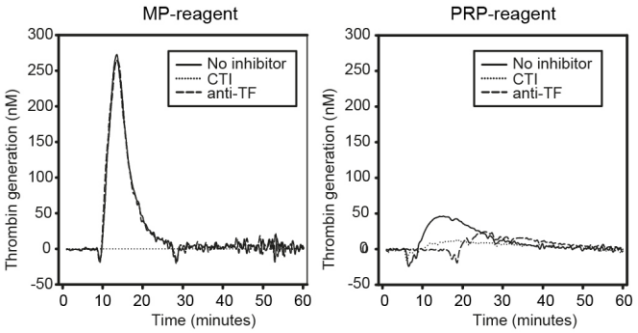


Figure 1: Limited activation of factor XII boosts thrombin generation in the presence of phospholipid membranes. Thrombin generation in platelet-free plasma using different combinations of kaolin and tissue factor in the presence and absence of phospholipids. The thrombin generation curves are average values from five donors.



	Inhibitor	Thrombin generation parameters			
		Lagtime (min)	ETP (nM*min)	Peak (nM)	ttPeak (min)
MP-reagent	-	11.4±1.2	1561±419	346±76	13.4±1.3
	anti-TF	11.0±1.2	1559±488	340±79	13.0±1.3
	CTI	0±0*	0±0*	0±0*	0±0*
PRP-reagent	-	9.2±1.3	673±277	46±16	15.6±1.3
	anti-TF	23.9±5.7	351±419	32±25	30.6±8.0
	CTI	22.5±14.1	209±346	18±19	32.4±18.5

Figure 2: Thrombin generation in plasma with MP-reagent is highly factor XII-dependent. Typical curves for plasma with the addition of MP-reagent (left) and PRP-reagent (right) either untreated or in the presence of FXII inhibitor (CTI, 100 µg/ml) or TF- blocking antibody (100 µg/ml). The table shows mean±SD, n=4. *Flat curve, not calculated by software.

Conflicts of interest

None of the authors have any conflicts of interest in relation to this study.

Addendum

N. Boknäs designed the research, performed experiments, interpreted data and wrote the manuscript. L. Faxälv conceived and designed the research, performed experiments and interpreted data. T.L. Lindahl made critical revision of the manuscript. S. Ramström conceived and designed the research, interpreted data and wrote the manuscript.