Venous thrombosis in cancer patients: Prediction, diagnosis and management
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Citation for published version (APA):

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Download date: 07 Jan 2019
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Measuring the tissue factor- and phospholipid-dependent properties of extracellular vesicles in their natural environment: the fibrin generation test

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Submitted
ABSTRACT

Extracellular vesicles support coagulation by providing negatively charged phospholipids, thereby enabling the formation of tenase and prothrombinase complexes, and by exposing tissue factor (TF). The presence of TF-exposing vesicles in blood is associated with several thrombotic diseases, including disseminated intravascular coagulation and venous thromboembolism, and requires reliable methods to measure the procoagulant properties of these vesicles. The aim of this report is to describe the characteristics and validation of a fibrin generation test, a one stage plasma clotting test, which measures the presence of procoagulant phospholipids as well as procoagulant TF on the vesicles in blood or other body fluids.
INTRODUCTION

The clotting of whole blood and plasma has been studied for over 200 years. Already in 1770 it was observed that blood collected from a healthy person completely coagulated in about 7 minutes, and that blood from patients with some diseases did not coagulate within half an hour (1). In 1878, Vierordt introduced the whole blood clotting time (WBCT). In this assay, a droplet of blood was collected in a capillary. The capillary was kept at 37°C and at 15 seconds time intervals a horse hair was inserted to assess whether a clot had been formed. The WBCT of a single person, measured daily for more than 50 days, ranged between 5-15 minutes. Obviously, the long clotting times and extensive variation made this assay unsuitable for routine clinical applications (2). It was not until 1914 that Howell developed a test to measure the clotting time (CT) of plasma. He mixed human blood with the anticoagulant sodium oxalate and then centrifuged this mixture to obtain plasma. Recalcification of plasma resulted in clotting, with observed CT values for healthy persons ranging between 9 – 12 minutes (3). Thereafter, oxalate was most replaced by sodium citrate, and most if not all plasma CT measurements were performed by adding an activator of either the intrinsic (factor XII-mediated) or extrinsic (factor VII-mediated) route of coagulation. These assays are now widely known as the activated partial thromboplastin time (aPTT) and prothrombin time (PT), respectively, and have largely replaced other plasma recalcification tests.

Nevertheless, the assay developed by Howell is intriguing because he measured the ability of plasma to clot after recalcification without addition of any activator of either factor XII or VII. Already in 1946 Chargaff and West showed that cell-free plasma contains a factor that promotes coagulation which was smaller than platelets, and that “the first appearance of fibers and the formation of a clot” in plasma were highly sensitive to removal by centrifugation prior to recalcification. In fact, we now know that plasma fails to clot when the plasma is recalcified after ultracentrifugation (4). This procedure results in complete removal of the subcellular factor and illustrates that the presence of this factor, the vesicles, is essential for the clotting of plasma.

Finding ways to measure the coagulant activity of vesicles has become important, because vesicles are increasingly ascribed direct roles in diseases such as venous thromboembolism and disseminated intravascular coagulation and therefore have the potential to become prognostic or diagnostic biomarkers. However, the contribution of vesicles to the clotting of plasma is complex. Vesicles provide phospholipids, especially phosphatidylserine, and thereby serve as a scaffold for the assembly of complexes of clotting factors. Next, vesicles can expose tissue factor (TF), the initiator of the coagulation process. Moreover, vesicles can directly activate factor XII (5;6), promote the formation of activated protein C, and support the conversion of plasminogen into plasmin (7;8). Therefore, studying vesicles directly in plasma is likely to provide a realistic insight into the contribution of vesicles to various aspects of the coagulation process. Measuring the ability of vesicles to promote clotting directly in plasma prevents the loss of subpopulations of vesicles by isolation procedures, circumvents
manipulation artifacts such as changes in the phospholipid distribution between the lipid bilayer, and does not require the addition of an excess of a strong coagulation activator such as kaolin or thromboplastin. These additions are intended to determine a clotting factor deficiency and therefore force coagulation reactions into one direction, and thus completely overshadow the putative contribution of vesicles to coagulation.

We previously published results of the fibrin generation test (FGT), a plasma recalcification test, which measures the coagulant phenotype of the vesicles, and does not require inhibitors of fibrin polymerization or removal of fibrin, isolation of vesicles, or the use of a chromogenic substrate or purified coagulation factors (4;9). Furthermore, because this assay is directly performed in plasma, vesicles remain in their natural physiological environment containing usually excessive levels of coagulation factors and inhibitors. Because the clotting time of this assay completely depends on the presence of the vesicles, this assay offers an easy and reliable tool to investigate the coagulant phenotype of vesicles. In this report, we describe the FGT in detail as an assay to measure the ability of vesicles to promote coagulation.

**MATERIALS AND METHODS**

**Antibodies and reagents**

Anti-factor VII (1.29 mg/ml, clone CLB VII-1) and anti-factor XII (1.035 mg/ml, OT-2) were obtained from Sanquin (Amsterdam, The Netherlands). Lactadharin (0.54 mg/ml) was obtained from Haematologic Technologies Inc. (Essex Junction, VT).

**Collection of blood**

Citrate-anticoagulated blood was collected from healthy subjects by venipuncture without a tourniquet through a 21-gauge needle using a Vacutainer system (BD 388607). The first tube of blood was discarded. Blood was collected in 3 ml plastic Vacutainer tubes (BD 363048, 0.0109 mol/l trisodium citrate f.c.) or 5 ml glass Vacutainer tubes (BD 367714, 0.0105 mol/l trisodium citrate f.c., BD Diagnostics; Franklin Jakes, NJ). All blood samples were centrifuged (20 minutes at 1,550 x g at 20°C) within 30 minutes after collection to reduce the risk of platelet activation, decay of clotting factors or inhibitors, generation of vesicles, etc., to prepare essentially platelet-free plasma. The upper two-third volume of the plasma was aspirated, collected in plastic 5 ml polystyrene tubes (BD 352052), and then briefly (< 5 s) mixed by vortex. In some experiments, normal pool plasma was used. The normal pool plasma samples each comprise plasma collected from more than 100 healthy subjects, both males and females, which are used as reference plasma for coagulation studies. These pool plasma samples are frozen as 500 µl aliquots and stored at -80°C.

**Fibrin generation test**

Recently, we have applied the fibrin generation test to measure the procoagulant properties of vesicles in saliva or as a tool to predict venous thromboembolism in oncological patients.
The fibrin generation test

In this assay, citrate-anticoagulated plasma (90 µl), essentially cell-free but containing the extracellular vesicles, is incubated in duplicate with either saline or inhibitory antibodies against either VIIa or XIIa, or lactadherin (all 3 µl) for 5 minutes at 37°C in a 96-well plate (med binding 650101; Greiner Bio-One, Monroe, NC). Fibrin generation is initiated by recalcification of the plasma. At t=0, CaCl₂ is added (15 µl of 0.1 mol/l, providing 13.9 mmol/l f.c. unless indicated otherwise. As a control, the plasma is subjected to ultracentrifugation to remove extracellular vesicles (1 hour at 154,000 x g and 4°C). Fibrin (clot) formation is monitored by measuring the optical density of the plasma at λ=405 nm on a Spectramax microplate reader (Molecular Devices Corp.; Sunnyvale, CA) at 37°C for 3,600 s. Because most plasma samples of healthy subjects have CT values ranging between 800 to 3,600 s, we arbitrarily decided to shake the ELISA plate every 60 s to reduce the risk of inhomogeneity.

The contribution of coagulant TF-exposing vesicles to coagulation is determined using an inhibitory antibody against human factor VII(a). The activity and specificity of anti-factor VII(a) has been extensively tested and described in normal plasma (4;10), and we prefer to use anti-factor VII(a) because this antibody can be used at a lower concentration (1.0 µg/ml f.c.) than anti-TF (7.8 µg/ml f.c.) to completely inhibit the TF-initiated activation of the coagulation process. Because the optical density (OD) values differ between plasma samples before the onset of coagulation, all measured values after recalcification are corrected for baseline, i.e. the OD at the start of the measurement. The outcome measure of the FGT, the clotting time (CT), is the time at which half of the maximum amount of fibrin has been formed (1/2 OD max).

Statistics
Data showed a non-Gaussian distribution, and differences were analyzed by Mann Whitney test using GraphPad Prism software (version 5.01; La Jolla, CA).

RESULTS
Choice of tube and stability of collected plasma used for FGT
In a first series of experiments, we compared the clotting time (CT), as monitored in the FGT and expressed as time at half maximum OD (½ OD max), of blood collected in glass or plastic tubes and immediately processed after blood collection. The CT of blood collected in glass tubes was significantly faster compared to plastic tubes, same subjects, 994 ± 283 vs. 2,118 ± 1,105 s (n=6; P=0.0183), respectively. To minimize the risk of the evident contact activation of the coagulation cascade by contact between blood and glass, plastic collection tubes were used for all future experiments involving the FGT.

Because several coagulation proteins deteriorate in time, especially at room temperature (11), we determined the stability of the collected plasma samples at room temperature and on melting ice. Blood from two subjects was collected and immediately processed to plasma. The FGT was measured in triplicate immediately after preparation of plasma and after storage of plasma at room temperature or on melting ice. As shown in
Figure 1, the CT of plasma samples from both subjects increased considerably already after one hour of storage at room temperature. In contrast, when plasma was stored on melting ice before the FGT was performed, the CT only modestly increased. Taken together, based on these results it's recommended to store plasma on melting ice for a maximum of 2 hours before performance of the FGT.

**Titrating CaCl\(_2\)**

Because the FGT is initiated by addition of CaCl\(_2\), the optimal concentration of CaCl\(_2\) was determined in plasma from two healthy subjects. As shown in Figure 2, plasma did not clot when concentrations of CaCl\(_2\) less than 8 mmol/l were added. Thus, up to this concentration of (added) CaCl\(_2\) insufficient free calcium ions are available to support the clotting of plasma. Final concentrations of CaCl\(_2\) ranging between 11 and 16 mmol/l are optimal, and CT again increase at higher concentrations of CaCl\(_2\). Therefore, in future experiments we arbitrarily use a final concentration of 13.9 mmol/l CaCl\(_2\).

**CT of healthy subjects: normal range of FGT**

Using the conditions described above, i.e. plastic blood collection tubes and final concentrations of 13.9 mmol/l CaCl\(_2\), blood samples from 22 healthy subjects (age 38.2 ± 9.5 y; 7 male) were collected. The mean CT as measured by the FGT was 1299 s (range 666 – 3,600 s).

**Effect of a single freeze-thaw step**

Subsequently, the FGT in plasma from healthy subjects (n=10), was measured immediately after collection of blood and preparation of plasma, and after a single freeze thaw step of plasma. The healthy subjects included were randomly selected from the 22 healthy subjects. The mean CT of fresh plasma was 999 s (range 559 – 1,644 s). The CT was unaffected by the single freeze-thaw cycle (mean 881 s, range 519 – 3,600 s; p=0.24).

**Dependence of the FGT on TF and phospholipids**

To investigate whether the FGT is capable of measuring TF-initiated coagulation, we prepared vesicle-free normal pool plasma and mixed the vesicle-free plasma 9 : 1 (v/v) with vesicle-containing human saliva, known to contain high numbers of coagulant TF-exposing vesicles, as described previously (4). The CT of vesicle-free normal pool plasma is > 3,600 s (data not shown). Addition of the 10% saliva shortens the CT from > 3,600 s to 338 ± 120 s (mean ± SD). In the presence of lactadherin, saliva-induced shortening of the CT is completely abolished, illustrating that coagulation cannot occur when lactadherin prevents the binding of coagulation factors to the phospholipid surface of the vesicles. Furthermore, as shown previously (4), inhibition of human factor VII(a) completely inhibits coagulation whereas preincubation with anti-factor XII has no effect. Thus, the FGT is also capable of measuring the contribution of coagulant, vesicle-associated TF.

Similarly, the dependency of the FGT on phospholipids and TF in plasma samples of normal healthy subjects (n=5) was determined. Again, the CT of vesicle-depleted plasma
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is > 3,600 s. Figure 3B shows that the CT of plasma is completely inhibited by lactadherin, not inhibited by anti-factor VII(a) and, for this particular subject, also unaffected by anti-factor XII. For 5 subjects, the CT was 673 s (625 – 805), which increased to 3600 s in the presence of lactadherin for all subjects (p=0.001 versus saline control), and 788 s (698 – 860, p=0.111) and 725 s (682 – 756, p=0.345) in the presence of anti-factor VII(a) or anti-factor XII. Although providing statistically non-significant prolongations in these experiments, the data indicate that the FGT can be used to measure extrinsic (TF-dependent) as well as intrinsic (factor XII-dependent) coagulation activation.

Reproducibility of the FGT

Subsequently, the intra- and inter-assay variation coefficient (VC) of the FGT was assessed. Because we and others are interested in the presence and contribution of coagulant TF-exposing vesicles in human plasma samples, the intra- and inter-assay VC using a mixture of 19 volumes of vesicle-free normal pool plasma and one volume of human pericardial “wound” blood (sample 1 in Table 1, i.e. plasma containing high levels of endogenous tissue factor (TF)-exposing and highly procoagulant vesicles (12)) as well as the 1:9 mixture of saliva and vesicle-depleted plasma were determined. As summarized in Table 1, the intra-assay of the CT was 276 s ± 13.5 s (mean ± SD; VC 4.9%; n=13), which increased 2.5-fold to 944 ± 93.5 s (VC 9.9%, n=13) in the presence of anti-VII. The inter-assay VCs were 11.7% and 17.6% (both n=30) in the absence or presence of anti-factor VII, respectively. Similarly, the intra- and inter-assay VCs for mixtures of cell-free human saliva, containing high levels of coagulant TF-exposing vesicles (4), and plasma in a 1:10 ratio were investigated. This mixture is called “sample 2” in Table 1. The intra-assay VC of the CT of sample 2 was 326 ± 11 s (n=9; VC 3.3%), which increased to > 3,600 s in the presence of anti-factor VII. The inter-assay VC was 8.7% in the absence of anti-VII and could not be determined in the presence of anti-VII (all results > 3,600s). Finally, the effect of anti-VII in normal pool plasma (Table 1, NPP 2007 and 2008) was measured. High mean clotting times (1175 – 1343 s) and thus large VC (25 – 28%) were observed.

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1Clotting time (s) in the presence of saline (control); 2clotting time in the presence of anti-factor VII(a); 3A 1:19 mixture of pericardial wound blood, containing coagulant TF-exposing vesicles, and vesicle-depleted normal pool plasma; 4A 1: 9 mixture of saliva containing coagulant TF-exposing vesicles and vesicle-depleted normal pool plasma; 5NPP: normal pool plasma prepared in 2007 and 2008.
DISCUSSION

In this study, we established the fibrin generation test as an assay to study the overall coagulant properties of cell-derived vesicles, as well as the specific contribution of TF-exposing vesicles to coagulation. Several aspects of the FGT require comment in comparison to other commonly used plasma clotting tests. The thrombin generation assay requires the use of a chromogenic substrate and removal of fibrinogen or the addition of a compound that blocks fibrin polymerization (10;13). The PPL assay, which measures the presence of procoagulant phospholipids, requires addition of factor Xa and cannot measure TF-dependent clotting (14). Capture (ELISA) assays only bind subpopulations of vesicles, requires addition of purified clotting factors and chromogenic substrate, and is unsuitable to measure single (fresh) samples (15-17). Finally, the factor Xa generation assays require isolation of vesicles and addition of both purified clotting factors and a chromogenic substrate, and is therefore very labor intensive (18). The present assay,
The fibrin generation test (FGT) requires only a spectrophotometer and inhibitory antibodies, can measure fresh as well as thawed samples, is cheap and easy to perform.

Essentially, the FGT described is a plasma recalcification assay as has been used for at least several decades. Previously, such assays were unsuitable for use in clinical practice because the CTs measured are slow and the reproducibility was poor (19;20). Why does the FGT work, whereas previously plasma recalcification assay did not? In the old recalcification tests, plasma usually contained vesicles, and the presence and contribution of phospholipids and especially coagulant TF may have markedly affected such measurements, and thus explain their poor reproducibility.

The reproducibility of the FGT is good in plasma with TF-initiated clotting. Such plasma samples have a fast CT. The reproducibility is only moderate in plasma samples that do contain no or hardly any TF-exposing vesicles. In such plasma samples, the clotting of the plasma is much more a balance between pro- and anti-coagulant processes, and fluctuation in such CTs is therefore likely to be much larger, as observed in our study.

Recently, we assessed the value of the FGT for the prediction of venous thrombosis in cancer patients in a pilot study, and compared the outcome to results obtained by PPL assay and Xa generation assay (9). The performance of the FGT was comparable to the Xa generation assay, but, as mentioned before, the FGT is easier to perform. At present, the FGT is used to study the coagulant properties of vesicles in cancer patients. This study is performed in 8 different hospitals, and over 600 patients have been included. In these hospitals, the test was performed by routine laboratories, often between measurements.
for patient care, showing that this assay can be implemented in other hospitals. To which extent this assay will be of clinical relevance, however, remains to be established.

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<tr>
<td>1. Hewson W. On the degree of heat which coagulates the lymph, and the serum of the blood; with an inquiry into the causes of the inflammatory crust, or size. as it is called. Phil Trans London 1770;60:384–97.</td>
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