Platelet activation and microparticles in the pericardial cavity during cardiopulmonary bypass
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Chapter 5

Pro- and non-coagulant forms of non-cell bound tissue factor in vivo

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ABSTRACT

**Background.** Concentrations of non-cell bound (‘soluble’) tissue factor (TF) are elevated in blood collecting in the pericardial cavity of patients during cardiopulmonary bypass (CPB). Previously, we reported microparticles supporting thrombin generation in such blood samples. In this study we investigated the extent of microparticle-association of the non-cell bound form of TF in pericardial and systemic blood, and whether this microparticle-associated form is active in thrombin generation as compared to non-microparticle bound, i.e. fluid-phase, TF.

**Methods.** Systemic and pericardial blood samples were collected before and during CPB from six patients undergoing cardiac surgery. Microparticles were isolated by differential centrifugation and their thrombin generating capacity measured in a chromogenic assay. Microparticle-associated and fluid-phase forms of non-cell bound TF were measured by ELISA. Microparticle-associated TF was visualized by flow cytometry.

**Results.** Forty-five to seventy-seven percent of non-cell bound TF was microparticle-associated in pericardial samples and triggered factor VII-mediated thrombin generation in vitro. Microparticles from systemic samples triggered thrombin generation independent from factor VII, except at the end of bypass (p=0.003). The fluid-phase form of TF did not initiate thrombin generation. Both forms of non-cell bound TF were –at least in part– antigenically cryptic.

**Conclusions.** We demonstrate the occurrence of two forms of non-cell bound TF. One form, which is microparticle-associated, supports thrombin generation via factor VII. The other form, which is fluid-phase, does not stimulate thrombin formation. We hypothesize that the microparticle-associated form of non-cell bound TF may be actively involved in postoperative thrombo-embolic processes when pericardial blood is returned into the patients.
INTRODUCTION

Administration of heparin during cardiopulmonary bypass (CPB) surgery does not preclude coagulation activation, as evidenced by elevated concentrations of thrombin-antithrombin complexes (TAT) and prothrombin fragment F1+2 in the systemic circulation.1-3 Previously, contact of blood with the extracorporeal circuit was thought to activate the intrinsic pathway coagulation.4 There is increasing evidence, however, for involvement of tissue factor (TF)–factor VII-dependent coagulation activation, the extrinsic pathway, especially in blood collecting in the pericardial cavity during CPB. Compared to systemic blood, pericardial blood contains highly elevated concentrations of F1+2, TAT and activated factor VII (VIIa), reflecting its highly activated coagulation state.1,5-6 To minimise blood loss, pericardial blood is returned into the patient, but this may activate systemic coagulation due to the presence of TF and factor VIIa.

Factor VII-dependent coagulation is initiated by TF, a transmembrane receptor for factor VII/VIIa. Normally, cellular TF is not present within the blood but extravascular in for example fibroblasts and smooth muscle cells.7,8 In pathological circumstances TF can be expressed by monocytes and endothelial cells in vitro and possibly in vivo. Low concentrations of non-cell bound TF are present in plasma, both in plasma from healthy individuals and at increased concentrations in for example malignant diseases,9 angina pectoris10 or disseminated intravascular coagulation (DIC).11 This non-cell bound TF is unlikely to initiate coagulation if truly ‘soluble’, because TF requires membrane association to become procoagulant.12

Recently, concentrations of non-cell bound TF were shown to be elevated in pericardial blood.13 These authors hypothesized that part of the non-cell bound TF may be associated with the cell-derived microparticles which we reported previously to be present in pericardial blood.14 These microparticles initiated thrombin generation that was completely inhibited by tissue factor pathway inhibitor (TFPI) and strongly delayed in factor VII-deficient plasma, and therefore likely to expose TF on their surface. More recently, we showed that microparticles from peripheral blood of a patient with meningococcal disease and extensive diffuse intravascular coagulation exposed TF and initiated thrombin generation via the extrinsic pathway in vitro.15
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In the present study we investigated the extent of microparticle-association of the non-cell bound form of TF in pericardial blood from patients undergoing CPB and its capability to support thrombin generation as compared to the fluid-phase form of non-cell bound TF. We also investigated whether microparticle-associated TF may explain the hypercoagulation found in the systemic circulation after return of the pericardial blood into the patient.

MATERIAL AND METHODS

Clinical studies. This study was approved by the ethical committee of the Onze Lieve Vrouwe Gasthuis. Six patients undergoing elective coronary artery bypass grafting with the use of CPB entered the study after their informed consent. Patients older than 85 years were excluded, and those with severe heart failure, renal or hepatic dysfunction, or a bleeding diathesis. Patients did not receive coumarin derivatives, aspirin, diprydamole or other nonsteroidal anti-inflammatory drugs within 5 days before the operation. Aprotinin or other antifibrinolytics were not used during the operation. CPB procedure and anaesthesia were similar as described before, as was the treatment of the patients. Heparin (3 mg/kg) was given intravenously before cannulation of the aorta and repeated in a dose of 50 mg whenever the activated clotting time (Hemocron; International Technidyne Corp., Edison, NJ) was shorter than 480 s.

Collection of blood samples. All systemic blood samples were drawn from the same central venous line. Blood samples from the pericardial cavity were taken directly with a 10 mL syringe. Sampling points of systemic blood (S) were after induction, before skin incision (S1), 5 minutes after start of CPB (S2), 10 minutes before release of the aortic crossclamp, at the start of the last distal anastomosis (S3), and before protamine administration (S4). Pericardial samples (P) were collected at points 2 through 4. Blood was immediately put into plastic tubes containing $1/10^{th}$ volume of 3.2% trisodiumcitrate (BD; San Jose, CA). Blood cells were removed by centrifugation for 20 minutes at 1,550 g at room temperature. Plasma samples were stored as 250 μL aliquots at -80°C.
Reagents and assays. Reptilase was obtained from Roche (Basel, Switzerland). Normal mouse serum was derived from Sanquin, Business Unit Reagents, at the CLB (Amsterdam, The Netherlands), anti-glycophorin A-PE (JC159, IgG₁) from DAKO (Glostrup, Denmark), anti-CD61-PE (VI-PL2, IgG₁) from PharMingen (San Jose, CA), annexin V-APC from CALTAG Laboratories (Burlingame, CA), IgG₁-FITC and IgG₁-PE (both X40) from BD, and anti-TF-FITC (4508CJ, IgG₁) from American Diagnostics, Inc. (Greenwich, CT). OT-2 (0.71 mg/mL), a monoclonal antibody (MoAb) inhibiting factor XII activity, has been described earlier. MoAbs directed against factor XI (clone XI-1) and factor VII (clones VII-1 and VII-15) activities were also from Sanquin.

Triton X-100 (Baker; Deventer, The Netherlands) was prepared as 0.5% stock-solution in phosphate-buffered saline (PBS; 154 mmol/L NaCl, 1.4 mmol/L phosphate, pH 7.4) containing 10.9 mmol/L trisodiumcitrate.

Plasma concentrations of F₁₋₂, TAT (Behring Diagnostics GmbH; Marburg, GER), IgG (CLB; Amsterdam, The Netherlands) and non-cell bound TF (American Diagnostics Inc.; Greenwich, CT, USA) were determined by ELISA according to manufacturers instructions. The standard curve of the non-cell bound TF ELISA was unaffected by 0.05% (v/v) Triton X-100 (data not shown). Concentrations of F₁₋₂, TAT and non-cell bound TF in samples S2-4 and P2-4 were corrected for hemodilution using their IgG content compared to that in S1.

Isolation of microparticles. Microparticles were isolated by differential centrifugation as described previously. Briefly, the microparticles were pelleted from the 250 μL plasma aliquots by centrifugation (see Collection of blood samples) for 30 minutes at 17,570 g and 20°C. Subsequently, 0.9 volume of the supernatant was removed, the microparticles were resuspended and washed once with PBS containing 10.9 mmol/L trisodiumcitrate. Finally, the microparticles were resuspended in 100 μL of the PBS/citrate buffer.

Flow cytometric analysis. Microparticles (5 μL) were diluted in 35 μL PBS containing 2.5 mmol/L CaCl₂ (pH 7.4) and 5 μL of 500-fold prediluted (in PBS) normal mouse serum, in the absence or presence of Triton X-100. Microparticles were stained with (i) annexin V-APC (5 μL), anti-TF-FITC (5 μL) and anti-CD61-PE (5 μL) or anti-glycophorin A-PE (5 μL), or (ii) annexin V-APC plus FITC- and PE-labeled IgG₁ control
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antibodies to set the fluorescence thresholds. Subsequently, microparticles were prepared and flow cytometry was performed on a FACSCaliber (BD; San Jose, CA), essentially as described previously.\textsuperscript{18}

**Thrombin generation test.** The thrombin generation test (TGT) was performed as described previously with minor modifications.\textsuperscript{15,20} Patient samples were treated with Hepzyme (Dade Behring GmbH; Marburg, GER) for 15 minutes at ambient temperature to remove heparin before microparticle isolation. Hepzyme (E.C. 4.2.2.7) was dissolved in 100 μL PBS, and control experiments showed that 2.5 μL of this solution degraded up to 5 U/mL (final concentration) unfractionated heparin (data not shown). Pefachrome TH-5114 (Pentapharm Ltd.; Basel, Switzerland) was used as chromogenic substrate to monitor thrombin activity. Concentrations and specificity of antibodies against factors VII, XI and XII were as described earlier.\textsuperscript{19} We did not perform anti-TF studies, because previously we observed no differences between anti-VII and anti-TF effects in the thrombin generation assay.\textsuperscript{15,19} Thrombin activity, expressed as nmol/L, was calculated with a reference curve of purified human α-thrombin (Sigma; St. Louis, MO, USA).\textsuperscript{19} For quantitative analysis, thrombin generation results were determined as area under the curve (AUC, 0-15 minutes after addition of CaCl\textsubscript{2}). Thrombin generation curves were not corrected for the dilution factor of the pericardial blood, because the extent at which the AUC is directly proportional to dilution, is not known.

**Data analysis.** Data were analysed with SPSS for Windows, release 9.0 (SPSS, Inc.; Chicago, Ill). Differences between all systemic and pericardial samples were evaluated by GLM univariate variation analysis at overall significance level of $p=0.05$, followed by post-hoc analysis (Scheffé test). The Mann Whitney U test was used to compare data obtained in the non-cell bound TF ELISA in the absence and presence of Triton X-100. Data are presented as median with range, unless indicated otherwise.

**RESULTS**

**Coagulation activation status and non-cell bound TF.** In the systemic circulation the concentrations of $F_{1+2}$ increased gradually from 0.8 nmol/L in S1 to 2.7
Figure 1. In vivo coagulation activation and concentrations of non-cell bound TF. Median concentrations of \( F_{1+2} \), TAT and non-cell bound TF (n=6) are shown in systemic (figures A, C and E, respectively) and in pericardial plasma samples (B, D and F, respectively). Collection points are indicated by systemic (S) 1-4 and pericard (P) 2-4. Note that y-axis are different between the systemic (open bars) and pericardial (gray bars) graphs; *p=0.05, **p=0.01 compared to S1.

nmol/L at the end of CPB in S4 (figure 1A). TAT concentrations also gradually increased from 2.0 µg/L in S1 to 13.4 µg/L in S4 (figure 1C). All concentrations of \( F_{1+2} \) and TAT in pericardial samples were higher than the highest concentration measured in the systemic samples. Compared to S1, the concentrations of \( F_{1+2} \) were 75-fold increased in P3 and 66-fold in P4 (figure 1B), and TAT concentrations 220-fold and 330-fold increased at these collection points, respectively (figure 1D). The increases were statistically significant in P3 (\( F_{1+2} \) p=0.013; TAT p=0.047 versus S1). Concentrations of non-cell bound TF were similar in all routinely prepared systemic plasma samples (figure 1E). Compared to S1
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(100 ng/L), the concentrations of non-cell bound TF were somewhat increased in P2 and P4 (173 and 293 ng/L, respectively), and significantly increased in P3 (1218 ng/L, p=0.048; figure 1F). Compared to systemic samples, the concentrations of non-cell bound TF were increased in P2 in 4 of the 6 patients, in P3 in all patients and in P4 in 5 of the 6 patients.

**Microparticle-associated TF.** To investigate whether non-cell bound TF was (partly) microparticle-associated, plasma samples were subjected to high-speed centrifugation and supernatant plasma as well as microparticle-containing pellets were assayed for non-cell-bound TF. In pericardial samples 21% (P2), 72% (P3) and 27% (P4) of TF was microparticle-associated (figure 2). TF concentrations in systemic samples were close to the detection limit and therefore too low to establish the microparticle-associated fraction of TF.

![Figure 2](image.png)

**Figure 2.** The microparticle-associated form of non-cell bound TF in pericardial samples. Collection points are indicated by pericard (P) 2–4.

**Thrombin generation by non-cell bound TF.** Pericardial microparticles elicited two types of thrombin generation curves. Either curves similar to figure 3A were obtained with a thrombin peak after approximately 3 minutes and a delay in thrombin generation with anti-factor VII. Alternatively, a gradually increasing thrombin generation was observed up to about 10 minutes, which was also delayed by anti-factor VII (figure 3B). Expressed as AUC, systemic microparticles generated less thrombin (figure 3C) than
Figure 3. Thrombin generation by microparticles in human plasma and coagulation pathways involved. Figures A and B show representative thrombin generation curves by microparticles from two patients both at P3, without antibodies (●) or in the presence of anti-factor XI (○), XII (□) or VII (Δ). Thrombin generation, expressed as AUC, by systemic and pericardial microparticles are shown in figure C and D (n=6); * p<0.01 compared to S1.

pericardial microparticles (figure 3D; P2 and P4 versus S1: p=0.009 and p=0.004, respectively). Because of the differences in thrombin generation curves, the inhibitory effects of anti-factor VII were both expressed as the extent of inhibition (figure 4A) and as the delay in onset (figure 4B) of thrombin generation. Anti-factor VII inhibited thrombin generation initiated by pericardial microparticles (P2: 38%, P3: 55% and P4: 28%; all p=0.001) or systemic microparticles from sampling point 4 (22%; p=0.003). Anti-factor VII also strongly delayed thrombin generation induced by pericardial microparticles (4, 5 and 3 minutes for microparticles from P2, P3 and P4, respectively, p<0.001), and approximately 1.5 minutes with microparticles from S4. Anti-factor XI and anti-factor XII did not substantially inhibit thrombin generation by pericardial microparticles (Table 1).
Figure 4. Inhibition of thrombin generation by anti-factor VII. Inhibition by anti-factor VII is presented as percentage inhibition of AUC (figure A) and delay in onset (figure B) of thrombin generation; * p<0.01 compared to S1.

Table 1. Inhibition of thrombin generation by anti-factor XI and XII. Percentage inhibition of the thrombin generation, expressed as AUC, from systemic (S1-4) and pericardial (P2-4) microparticles (n=6, median and range between brackets).

<table>
<thead>
<tr>
<th>Sample point</th>
<th>anti-factor XI (% inhibition)</th>
<th>anti-factor XII (% inhibition)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>15 (-2 - 20)</td>
<td>5.5 (-9 - 11)</td>
</tr>
<tr>
<td>S2</td>
<td>7 (-18 - 27)</td>
<td>0 (-11 - 19)</td>
</tr>
<tr>
<td>S3</td>
<td>16 (5 - 30)</td>
<td>-3.5 (-11 - 8)</td>
</tr>
<tr>
<td>S4</td>
<td>17 (15 - 22)</td>
<td>7.5 (-1 - 15)</td>
</tr>
<tr>
<td>P2</td>
<td>8 (-12 - 14)</td>
<td>11.5 (4 - 17)</td>
</tr>
<tr>
<td>P3</td>
<td>3 (-2 - 7)</td>
<td>-3 (-7 - 5)</td>
</tr>
<tr>
<td>P4</td>
<td>8.5 (-3 - 13)</td>
<td>10 (5 - 23)</td>
</tr>
</tbody>
</table>
The supernatants, which are microparticle-free systemic or pericardial plasma samples, obtained after removal of the microparticles but still containing substantial concentrations of the non-microparticle associated, i.e. fluid-phase, form of TF, were not capable of detectable thrombin generation (data not shown).

**Visualization of the microparticle-associated form of TF.** Compared to the control antibody (figure 5A), pericardial microparticles hardly stained for TF using flow cytometry (figure 5C). To improve the detection of TF, pericardial microparticles were incubated with increasing concentrations of Triton X-100 (0.01-0.10% v/v). The TF antigen became visible at concentrations between 0.02-0.08% (v/v) (data not shown).

![Image of flow cytometry plots showing detection of TF on pericardial microparticles with and without Triton X-100](image)

**Figure 5.** Detection of TF on pericardial microparticles. Flow cytometry dot plots are provided from one representative experiment. Microparticles from P3 were stained with control antibody IgG1-FITC (A, B) or anti-TF-FITC (C, D) in the absence (figures 5A, 5C) or presence (figures 5B, 5D) of Triton X-100.
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Arbitrarily, we used 0.05% of Triton X-100 in subsequent experiments. With Triton X-100, the staining of microparticles with control antibody was unaffected (figure 5B versus 5A), but the TF antigen became detectable (figure 5D). Triton X-100 impaired the binding of annexin V as well as antigens such as CD61 and glycophorin A (data not shown). Therefore, the cellular origin of TF-exposing microparticles could not be established.

**Total plasma concentrations of non-cell bound TF.** Because Triton X-100 improved the detection of the microparticle-associated form of non cell-bound TF by flow cytometry, we hypothesized that part of the non-cell bound TF in plasma may also remain undetected by ELISA. In pericardial samples, total concentrations of non-cell bound TF increased 1.4-3.1 fold with Triton X-100 (Table 2), but not in systemic samples (data not shown). This effect of Triton X-100 was most prominent in pericardial samples from collection points 2 and 4. As shown in Table 2, the detection of fluid-phase TF also increased in the presence of Triton X-100. Thus, the concentrations of both forms of non-cell bound TF, which are the microparticle-associated form as well as the fluid phase form, are underestimated in the absence of Triton X-100.

<table>
<thead>
<tr>
<th>Sample point</th>
<th>Triton X-100</th>
<th>P2</th>
<th>P3</th>
<th>P4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total non-cell bound TF, ng/L</td>
<td>173</td>
<td>96-728</td>
<td>216-2052</td>
<td>1218</td>
</tr>
<tr>
<td>MP-associated TF, ng/L</td>
<td>28</td>
<td>13-268</td>
<td>76-1053</td>
<td>233 *</td>
</tr>
<tr>
<td>Fluid-phase TF, ng/L</td>
<td>119</td>
<td>61-412</td>
<td>92-522</td>
<td>296 *</td>
</tr>
<tr>
<td>% MP-associated TF</td>
<td>21</td>
<td>16-41</td>
<td>35-68</td>
<td>45 *</td>
</tr>
</tbody>
</table>

* * p=0.028, ** p=0.043
DISCUSSION

In systemic blood TF can be exposed on cells and be present in a non-cell bound form. The present study shows that in pericardial plasma two forms of non-cell bound TF occur. One form is microparticle-associated, whereas the other form is not. This latter form we call fluid-phase TF, but it may be attached to albumin, lipoproteins, etc. So, in human blood TF can be present in a cell-bound form, a microparticle-associated form, and in a fluid-phase form. To which extent non-cell bound TF in plasma from healthy human individuals is identical to non-cell bound TF in plasma from patients undergoing cardiac surgery remains to be determined.

The present study shows that only the microparticle-associated form of non-cell bound TF generated thrombin. Previously, we showed that cell-derived microparticles in pericardial blood originate from platelets (46%), erythrocytes (43%), and possibly granulocytes (10%). Since Triton X-100 impaired their identification, at present we can only speculate which microparticles expose TF. Since TF has been found to be associated with platelets, granulocytes, or microparticles derived therefrom, we hypothesize that at least part of the microparticle-associated form of non-cell bound TF may be exposed by such microparticles in pericardial blood.\textsuperscript{21,22} Our findings with Triton X-100 demonstrate that TF is detectable on some microparticles but not on all, and it is not clear whether those TF-positive microparticles—as demonstrated by flow cytometry—are indeed all capable of thrombin generation. In fact, there is already some evidence suggesting that the procoagulant activity of microparticle-exposed TF may be dependent on the cellular origin of the TF-exposing microparticles. Whereas we previously showed that microparticles of monocytic origin exposed coagulant TF in a patient with meningococcal septic shock and severe disseminated intravascular coagulation, we more recently found increased numbers of especially platelet-derived microparticles exposing TF that were clearly not coagulant.\textsuperscript{15,27} Thus, at present, we cannot exclude that some differences in cellular origin of TF-exposing microparticles between plasma samples may affect the thrombin generation capacity. In an attempt to characterize the microparticle-associated form(s) of TF, we applied pericardial and systemic microparticles to Western blot. Staining by anti-TF only revealed a weak band of approximately 45 kDa in the pericardial
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microparticle fraction (data not shown), suggesting that at least part of TF present in these samples is of the full-length form. From these data, however, we cannot exclude that 'microparticle-associated TF' still may contain several forms of both active and/or inactive TF.

In the pericardial blood samples with the highest concentration of non-cell bound TF, TAT, and F$_{1+2}$, which is at sample point P3, up to 77% of the non-cell bound TF was in the microparticle-associated form. The other pericardial blood samples (P2 and P4), which are the blood samples showing much less in vivo coagulation activation than at P3, contained only 21 to 27% of the microparticle-associated form of TF. Despite the fact that there was considerably more microparticle-associated TF at P3 than in the 2 other pericardial samples, thrombin generation was comparable in vitro. Most likely, this is due to correction for hemodilution of the microparticle TF content to enable comparison to the similarly corrected TAT and F$_{1+2}$ concentrations. At sampling point P3, the correction factor for hemodilution is about 7 and the uncorrected concentrations of non-cell bound TF –i.e. the actual amount of TF that was added in the thrombin generation experiments, did not differ significantly from TF concentrations in samples collected at P2 and P4.

The factor-VII dependent coagulation pathway was involved in the thrombin generation of the microparticle-associated form of TF. This was shown by inhibition of thrombin generation by anti-factor VII, both expressed as the AUC and the delay in onset of thrombin generation. In most institutes for cardiosurgery, pericardial wound blood is returned into the systemic circulation during and especially at the end of the CPB procedure to reduce blood loss. We observed systemic coagulation activation in blood samples collected at the end of the bypass (S4), despite heparinization. Moreover, anti-factor VII inhibited thrombin generation only when initiated by systemic microparticles collected at the end of bypass. Therefore, we hypothesize that pericardial microparticles after their return into the circulation are responsible for the systemic coagulation activation.

It is tempting to speculate that the transfusion of the pericardial cavity blood, and thus of high numbers of procoagulant microparticles with the active TF on their surface, may contribute to the post-operative thrombotic complications such as graft occlusion, silent deep vein thrombosis and adverse neurological events in patients undergoing
cardiac surgery assisted by CPB.\textsuperscript{23-25} Bonderman et al. already showed in a porcine model that intracoronary injection of relipidated human TF induces coronary no-reflow by a thrombus.\textsuperscript{26}

In summary, our present findings show that non-cell bound TF may occur in a microparticle-associated and in a fluid-phase form in human plasma. The microparticle-associated form was capable of factor VII-mediated thrombin. In contrast, the fluid-phase form did not initiate thrombin generation. The contribution of pericardial blood to systemic coagulation activation may explain the coagulation activation at the end of the CPB. Furthermore, we hypothesize that in other clinical conditions,\textsuperscript{9-11} in which elevated concentrations of non-cell bound TF antigen in plasma were paralleled by an increased risk for thromboembolic events, the microparticle-associated form of non-cell bound TF is likely to contribute to systemic coagulation activation.

REFERENCES

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