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ORIGINAL ARTICLE

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

K. N. STURK-MAQUELIN,* R. NIEUWLAND,† F. P. H. T. M. ROMIJN,‡ L. EIJSMAN,* C. E. HACK§ and A. STURK†

*Department of Cardiothoracic Surgery, Onze Lieve Vrouwe Gasthuis, Amsterdam, The Netherlands; †Department of Clinical Chemistry, Academic Medical Center of the University of Amsterdam, Amsterdam, The Netherlands; ‡Department of Clinical Chemistry, Leiden University Medical Center, Leiden, The Netherlands; §Department of Immunopathology, Sanquin Research at the CLB, Amsterdam, The Netherlands; and Department of Clinical Chemistry, Medical Center Vrije Universiteit, Amsterdam, The Netherlands

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Summary. *Background*: Concentrations of non-cell-bound (NCB; 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 NCB form of TF in pericardial and systemic blood, and whether this microparticle-associated form is active in thrombin generation compared with non-microparticle-bound, (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 NCB TF were measured by ELISA. Microparticle-associated TF was visualized by flow cytometry. *Results*: In pericardial samples, 45–77% of NCB TF was microparticle-associated, and triggered factor VII (FVII)-mediated thrombin generation *in vitro*. Microparticles from systemic samples triggered thrombin generation independently of FVII, except at the end of bypass ($P = 0.003$). The fluid-phase form of TF did not initiate thrombin generation. Both forms of NCB TF were, at least in part, antigenically cryptic. *Conclusions*: We demonstrate the occurrence of two forms of NCB TF. One form, which is microparticle-associated, supports thrombin generation via FVII. The other form, which is fluid-phase, does not stimulate thrombin formation. We hypothesize that the microparticle-associated form of NCB TF may be actively involved in postoperative thromboembolic processes when pericardial blood is returned into the patients.

Correspondence: Rienk Nieuwland, AMC, Department of Clinical Chemistry, PO Box 22660, 1100 DD Amsterdam, The Netherlands.
Tel.: +31 20 566 4851; fax: +31 20 609 1222; e-mail: r.nieuwland@amc.uva.nl

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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 (FVII)-dependent coagulation activation (the extrinsic pathway), especially in blood collecting in the pericardial cavity during CPB. Compared with systemic blood, pericardial blood contains highly elevated concentrations of $F1+2$, TAT and activated FVII (FVIIa), reflecting its highly activated coagulation state [1,5,6]. To minimize blood loss, pericardial blood is returned into the patient, but this may activate systemic coagulation because of the presence of TF and FVIIa.

FVII-dependent coagulation is initiated by TF, a transmembrane receptor for FVII/FVIIa. Normally, cellular TF is not present within the blood, but is extravascular, e.g. in 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 (NCB) TF are present in plasma, both in plasma from healthy individuals and at increased concentrations in plasma from patients with disorders such as malignancy [9], angina pectoris [10] or disseminated intravascular coagulation (DIC) [11]. This NCB TF is unlikely to initiate coagulation if truly 'soluble', because TF requires membrane association to become procoagulant [12].

Recently, concentrations of NCB TF were shown to be elevated in pericardial blood [13]. These authors hypothesized that part of the NCB TF may be associated with the cell-derived microparticles that we reported previously to be present in pericardial blood [14]. These microparticles initiated thrombin generation that was completely inhibited by tissue factor pathway inhibitor and strongly delayed in FVII-deficient plasma, and therefore were likely to expose TF on their surfaces. More recently, we showed that microparticles from peripheral blood of a patient with meningococcal disease and extensive DIC exposed TF and initiated thrombin generation via the extrinsic pathway *in vitro* [15].

In the present study we investigated the extent of microparticle association of the NCB form of TF in pericardial blood from patients undergoing CPB and its capability to support thrombin generation compared with the fluid-phase form of NCB TF. We also investigated whether microparticle-associated TF may explain the hypercoagulation found in the systemic circulation after return of pericardial blood into patients.

Materials and methods

Clinical studies

This study was approved by the ethics 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, dipyridamole or other non-steroidal anti-inflammatory drugs within 5 days before the operation. Aprotinin or other antifibrinolytics were not used during the operation. CPB procedure and anesthesia were similar to those described previously, as was the treatment of the patients [16]. Heparin (3 mg kg^{-1}) was given intravenously before cannulation of the aorta and repeated in a dose of 50 mg whenever the activated clotting time (measured by Haemocron; International Technidyne Corp., Edison, NJ, USA) was shorter than 480 s.

Collection of blood samples

All systemic blood samples were drawn from the same central venous line. Systemic (S) blood samples were taken after induction, before skin incision (S1), 5 min after start of CPB (S2), 10 min before release of the aortic crossclamp, at the start of the last distal anastomosis (S3), and before protamine administration (S4). Blood samples from the pericardial cavity (P) were taken directly with a 10 mL syringe at points 2 through 4. Blood was immediately put into plastic tubes containing 10% volume of 3.2% trisodiumcitrate (BD; San Jose, CA, USA). Blood cells were removed by centrifugation for 20 min at 1550 *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, and mAbs directed against FXI (clone XI-1) and FVII (clones VII-1 and VII-15) activities were purchased from Sanquin, Business Unit Reagents, (CLB, Amsterdam, The Netherlands), antiglycophorin A-PE (JC159, IgG_1) from DAKO (Glostrup, Denmark), anti-CD61-PE (VI-PL2, IgG_1) from PharMingen (San Jose, CA, USA), annexin V-APC from CALTAG Laboratories (Burlingame, CA, USA), IgG_1-FITC and IgG_1-PE (both X40) from BD, and anti-TF-FITC (4508CJ, IgG_1) from American Diagnostics, Inc. (Greenwich, CT, USA). OT-2 (0.71 mg mL^{-1}), a monoclonal antibody (mAb) inhibiting FXII activity, has been described earlier [17]. .

Triton X-100 (Baker; Deventer, The Netherlands) was prepared as 0.5% stock-solution in phosphate-buffered saline (PBS; 154 mmol L^{-1} NaCl, 1.4 mmol L^{-1} phosphate, pH 7.4) containing 10.9 mmol L^{-1} trisodiumcitrate.

Plasma concentrations of $F1+2$, TAT (Behring Diagnostics GmbH; Marburg, Germany), IgG (CLB) and NCB TF (American Diagnostics Inc.) were determined by ELISA according to manufacturers' instructions. The standard curve of the NCB TF ELISA was unaffected by 0.05% (v/v) Triton X-100 (data not shown). Concentrations of $F1+2$, TAT and NCB TF in samples S2-4 and P2-4 were corrected for hemodilution using their IgG content compared with that in S1.

Isolation of microparticles

Microparticles were isolated by differential centrifugation as described previously [18,19]. Briefly, the microparticles were pelleted from the 250 μL plasma aliquots by centrifugation for 30 min at 17 570 *g* and 20 °C. Subsequently, 90% volume of the supernatant was removed, and the microparticles were resuspended and washed once with PBS containing 10.9 mmol L^{-1} 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^{-1} $CaCl_2$ (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 antiglycophorin A-PE (5 μL), or (ii) annexin V-APC plus FITC- and PE-labeled IgG_1 control antibodies to set the fluorescence thresholds. Subsequently, microparticles were prepared and flow cytometry was performed on a FACSCalibur (BD), essentially as described previously [18].

Thrombin-generation test

The thrombin-generation test (TGT) was performed as described previously with minor modifications [15,20]. Patient samples were treated with Hepzyme (Dade Behring GmbH;

Marburg, GER) for 15 min 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^{-1} (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 [19]. We did not perform anti-TF studies, because previously we observed no differences between anti-FVII and anti-TF effects in the thrombin-generation assay [15,19]. Thrombin activity, expressed as nmol L^{-1}, was calculated with a reference curve of purified human α-thrombin (Sigma; St. Louis, MO, USA) [19]. For quantitative analysis, thrombin-generation results were determined as area under the curve (AUC, 0–15 min after addition of $CaCl_2$). Thrombin-generation curves were not corrected for the dilution factor of the pericardial blood, because the extent of which the AUC is directly proportional to dilution is not known.

Data analysis

Data were analyzed with SPSS for Windows, release 9.0 (SPSS, Inc.; Chicago, IL, USA). Differences between all systemic and pericardial samples were evaluated by GLM univariate variation analysis at overall significance level of $P = 0.05$, followed by posthoc analysis (Scheffé test). The Mann–Whitney *U*-test was used to compare data obtained in the NCB 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 NCB TF

In the systemic circulation, the concentrations of *F*1+2 increased gradually from 0.8 nmol L^{-1} in S1 to 2.7 nmol L^{-1} at the end of CPB in S4 (Fig. 1A). TAT concentrations also gradually increased from 2.0 μg L^{-} in S1 to 13.4 μg L^{-} in S4 (Fig. 1C). All concentrations of *F*1+2 and TAT in pericardial samples were higher than the highest concentration measured in the systemic samples. Compared with S1, the concentrations of *F*1+2 were 75-fold increased at P3 and 66-fold at P4 (Fig. 1B), and TAT concentrations increased 220-fold and 330-fold at these collection points, respectively (Fig. 1D). The increases were statistically significant at P3 (*F*1+2 $P = 0.013$; TAT $P = 0.047$ vs. S1). Concentrations of NCB TF were similar in all routinely prepared systemic plasma samples (Fig. 1E). Compared with S1 (100 ng L^{-1}), the concentrations of NCB TF were somewhat increased at P2 and P4 (173 and 293 ng L^{-1}, respectively), and significantly increased at collection point 3

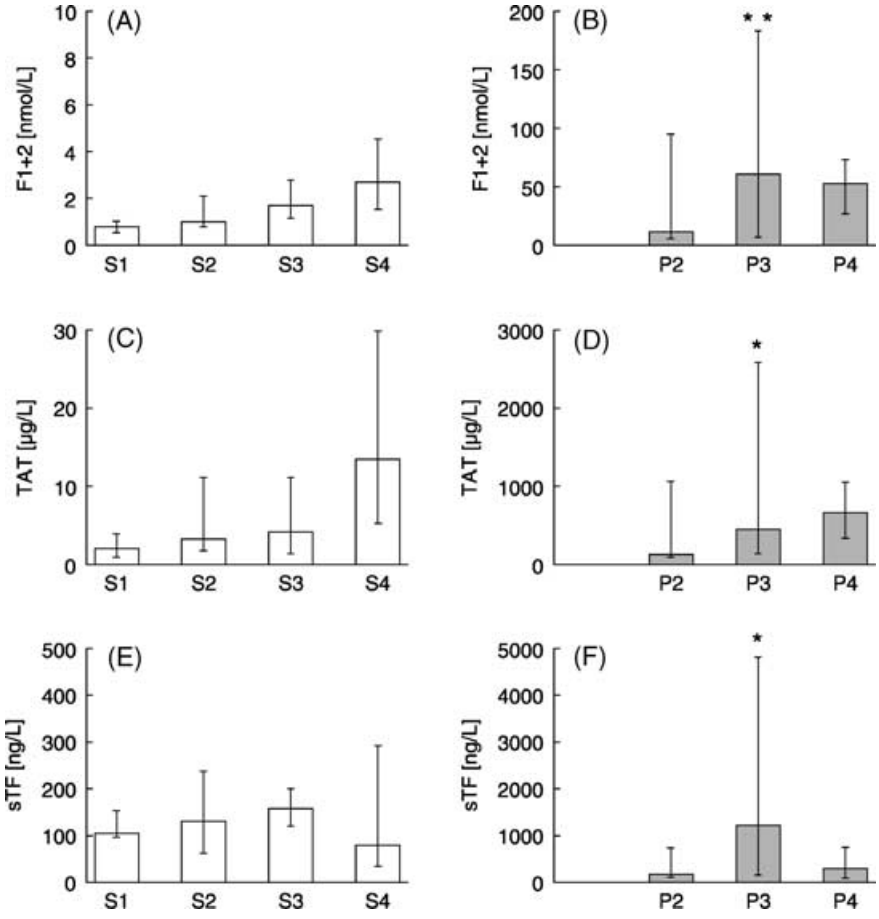

Fig. 1. *In vivo* coagulation activation and concentrations of NCB TF. Median concentrations of *F*1+2, TAT and NCB TF ($n = 6$) are shown in systemic (A, C and E, respectively) and in pericardial (B, D and F, respectively) plasma samples. Collection points are indicated by systemic (S) 1–4 and pericardial (P) 2–4. Note that *y*-axes are different between the systemic (open bars) and pericardial (gray bars) graphs; $^{*}P = 0.05$, $^{**}P = 0.01$ compared with S1.

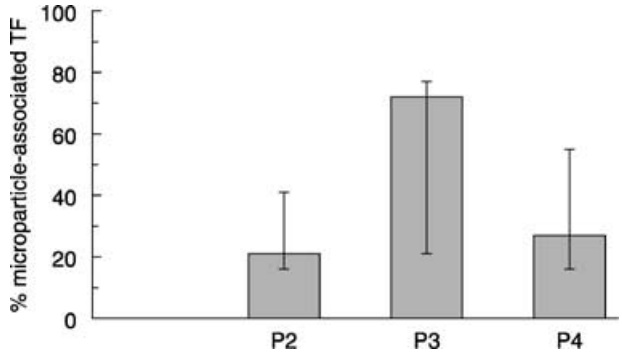

Fig. 2. The microparticle-associated form of NCB TF in pericardial samples. Collection points are indicated by pericardial (P) 2–4.

(1218 ng L^{-1}, $P = 0.048$; Fig. 1F). Compared with systemic samples, the concentrations of NCB TF were increased at P2 in 4/6 patients, at P3 in all patients and at P4 in 5/6 patients.

Microparticle-associated TF

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

Thrombin generation by NCB TF

Pericardial microparticles elicited two types of thrombin-generation curves. The first was similar to Fig. 3(A), obtained with a thrombin peak after approximately 3 min, and a delay in thrombin generation with anti-FVII. Alternatively, a gradually increasing thrombin generation was observed up to about 10 min, which was also delayed by anti-FVII (Fig. 3B). Expressed as AUC, systemic microparticles generated less thrombin (Fig. 3C) than pericardial microparticles (Fig. 3D; P2 and P4 vs. S1: $P = 0.009$ and $P = 0.004$, respectively). Because of the differences in thrombin-generation curves, the inhibitory effects of anti-FVII were expressed both as the extent of inhibition (Fig. 4A) and the delay in onset (Fig. 4B) of thrombin generation. Anti-FVII inhibited thrombin generation initiated by pericardial microparticles (P2: 38%, P3: 55% and P4: 28%; all $P = 0.001$) or systemic microparticles from P4 (22%; $P = 0.003$). Anti-FVII also strongly delayed thrombin generation induced by pericardial microparticles (4, 5 and 3 min for microparticles from P2, P3 and P4, respectively, $P < 0.001$), and approximately 1.5 min with microparticles from S4. Anti-FXI and anti-FXII did not substantially inhibit thrombin generation by pericardial microparticles (Table 1).

The supernatants, which are microparticle-free systemic or pericardial plasma samples, obtained after removal of the microparticles but still containing substantial concentrations of the fluid-phase form of TF, were not capable of detectable thrombin generation (data not shown).

Visualization of the microparticle-associated form of TF

Compared with the control antibody (Fig. 5A), pericardial microparticles hardly stained for TF using flow cytometry (Fig. 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 and 0.08% (v/v) (data not shown). 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 (Fig. 5B vs. 5A), but the TF antigen became detectable (Fig. 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.

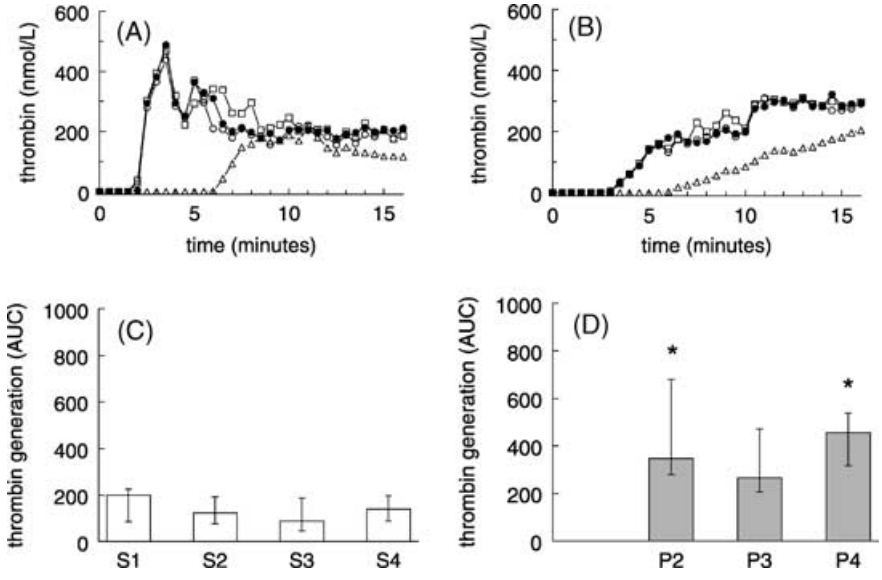

Fig. 3. Thrombin generation by microparticles in human plasma and coagulation pathways involved. (A, B) show representative thrombin-generation curves by microparticles from two patients both at P3, without antibodies (●) or in the presence of antifactor XI (□), XII (○) or VII (△). Thrombin generation, expressed as AUC, by systemic and pericardial microparticles are shown in (C, D) ($n = 6$); $^{*}P < 0.01$ compared with S1.

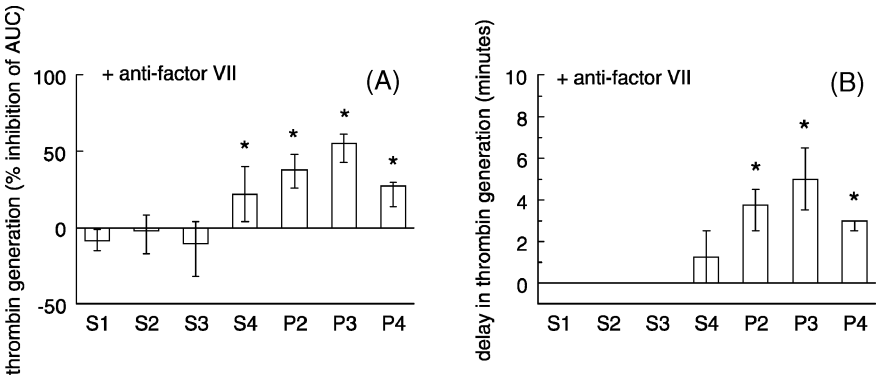

Fig. 4. Inhibition of thrombin generation by anti-FVII. Inhibition by anti-FVII is presented as percentage inhibition of AUC (A) and delay in onset (B) of thrombin generation; $^{*}P < 0.01$ compared with S1.

Table 1 Inhibition of thrombin generation by antifactor XI and XII. Percentage inhibition of the thrombin generation, expressed as AUC, from systemic (S1-4) and pericardial (P2-4) microparticles

	% inhibition	
Sample point	Antifactor XI [median (range)]	Antifactor XII [median (range)]
S1	15 (−2 to 20)	5.5 (−9 to 11)
S2	7 (−18 to 27)	0 (−11 to 19)
S3	16 (5 to 30)	−3.5 (−11 to 8)
S4	17 (15 to 22)	7.5 (−1 to 15)
P2	8 (−12 to 14)	11.5 (4 to 17)
P3	3 (−2 to 7)	−3 (−7 to 5)
P4	8.5 (−3 to 13)	10 (5 to 23)

$n = 6$. Median and (range).

Total plasma concentrations of NCB TF

Because Triton X-100 improved the detection of the microparticle-associated form of NCB TF by flow cytometry, we hypothesized that part of the NCB TF in plasma may also remain undetected by ELISA. In pericardial samples, total concentrations of NCB 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 NCB TF are underestimated in the absence of Triton X-100.

Discussion

In systemic blood, TF can be exposed on cells and be present in a NCB form. The present study shows that in pericardial plasma, two forms of NCB 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. Thus, 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 NCB TF in plasma from healthy human individuals is identical to NCB TF in plasma from patients undergoing cardiac surgery remains to be determined.

The present study shows that only the microparticle-associated form of NCB TF generates 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 from these, we hypothesize that at least part of the microparticle-associated form of NCB TF may be exposed by such microparticles in pericardial blood [21,22–26]. 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 DIC, we more recently found increased numbers of TF-exposing microparticles, especially platelet-derived microparticles, which were clearly not coagulant [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 western blotting pericardial and systemic microparticles. Staining by anti-TF revealed only a weak band of approximately 45 kDa in the pericardial 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 NCB TF, TAT, and $F1+2$, which was at sample point 3, up to 77% of the NCB TF was in the microparticle-associated form. The other pericardial blood samples (P2 and P4), which were the blood samples showing much less *in vivo* coagulation activation than P3, contained only 21–27% of the microparticle-

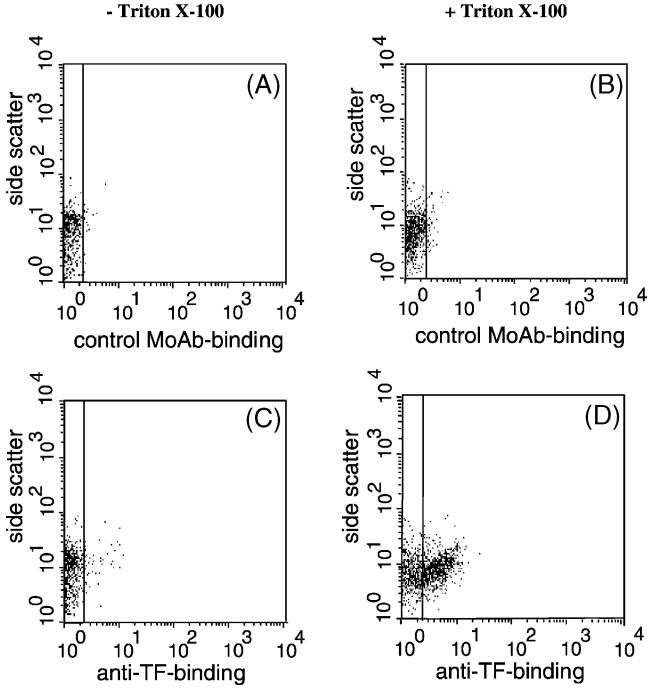

Fig. 5. Detection of TF on pericardial microparticles. Flow cytometry dot plots are provided from one representative experiment. Microparticles from P3 were stained with control antibody IgG_1-FITC (A, B) or anti-TF-FITC (C, D) in the absence (A, C) or presence (B, D) of Triton X-100.

associated form of TF. Despite the fact that there was considerably more microparticle-associated TF at P3 than in the two other pericardial samples, thrombin generation was comparable *in vitro*. Most likely, this is due to the correction for hemodilution of the microparticle TF content used to enable comparison with the similarly corrected TAT and $F1+2$ concentrations. At P3, the correction factor for hemodilution is about 7 and the uncorrected concentrations of NCB 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 FVII-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-FVII, 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-FVII inhibited thrombin generation only when initiated by systemic microparticles collected at the end of bypass. Therefore, we hypothesize that pericardial microparticles are responsible for the systemic coagulation activation after their return into the circulation.

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 postoperative thrombotic complications such as graft occlusion, silent deep vein thrombosis and adverse neurological events in patients undergoing cardiac surgery assisted by CPB [23–25]. However, we cannot exclude the possibility that increased cellular expression of TF may also play a role. For instance, monocytes in the oxygenator of the cardiopulmonary bypass circuit have an increased surface exposure of TF and these cells are also returned into the circulation, although increased TF expression could not be demonstrated on these cells in the systemic circulation [28]. Additionally, we cannot exclude the possibility that the contribution of microparticles is underestimated due to their binding to cells such as monocytes, granulocytes and platelets, thereby providing these cells with procoagulant TF and providing subsequently detached microparticles with antigens of blood cells other than their parent cell [27]. Bonderman *et al*. have already shown in a porcine model that intracoronary injection of relipidated human TF induces coronary no-reflow by a thrombus [26].

In summary, our present findings show that NCB TF may occur in a microparticle-associated and in a fluid-phase form in human plasma. The microparticle-associated form was capable of FVII-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 CPB. Furthermore, we hypothesize that in other clinical conditions [9–11], in which elevated concentrations of NCB TF antigen in plasma are paralleled by an increased risk for thromboembolic events, the microparticle-associated form of NCB TF is likely to contribute to systemic coagulation activation.

Table 2 Concentrations of NCB TF. The concentrations of NCB TF, as well as the concentrations of microparticle (MP)-associated and fluid-phase forms of (NCB) TF were determined in the absence or presence of Triton X-100. The percentage of the MP-associated form is the calculated percentage of the MP-associated plus fluid-phase form

Sample point	P2		P3		P4	
Triton X-100	−	+	−	+	−	+
Total NCB TF ($ng\,L^{-1}$)	173 (96–728)	537* (216–2052)	1218 (153–4811)	1744 (80–4967)	293 (86–743)	849* (414–3339)
MP–associated TF ($ng\,L^{-1}$)	28 (13–268)	233* (76–1053)	854 (55–2473)	1047 (82–3792)	67 (27–370)	411** (276–2142)
Fluid–phase TF ($ng\,L^{-1}$)	119 (61–412)	296* (92–522)	310 (206–972)	402 (9–1104)	164 (103–363)	225** (192–833)
MP–associated TF (%)	21 (16–41)	45* (35–68)	72 (21–77)	77 (71–90)	27 (16–55)	63** (59–72)

$n = 6$. Median and (range); $^{*}P = 0.028$, $^{**}P = 0.043$. NCB, non-cell-bound

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