

# Human cell-derived microparticles promote thrombus formation *in vivo* in a tissue factor-dependent manner

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**Summary.** *Background:* Circulating microparticles of various cell types are present in healthy individuals and, in varying numbers and antigenic composition, in various disease states. To what extent these microparticles contribute to coagulation *in vivo* is unknown. *Objectives:* To examine the *in vivo* thrombogenicity of human microparticles. *Methods:* Microparticles were isolated from pericardial blood of cardiac surgery patients and venous blood of healthy individuals. Their numbers, cellular source, and tissue factor (TF) exposure were determined using flow cytometry. Their *in vitro* procoagulant properties were studied in a fibrin generation test, and their *in vivo* thrombogenicity in a rat model. *Results:* The total number of microparticles did not differ between pericardial samples and samples from healthy individuals ( $P=0.786$ ). In both groups, microparticles from platelets, erythrocytes, and granulocytes exposed TF. Microparticle-exposed TF antigen levels were higher in pericardial compared with healthy individual samples ( $P=0.036$ ). Pericardial microparticles were strongly procoagulant *in vitro* and highly thrombogenic in a venous stasis thrombosis model in rats, whereas microparticles from healthy individuals were not [thrombus weights 24.8 (12.2–41.3) mg vs. 0 (0–24.3) mg median and range;  $P<0.001$ ]. Preincubation of pericardial microparticles with an inhibitory antibody against human TF abolished their thrombogenicity [0 (0–4.4) mg;  $P<0.01$ ], while a control antibody had no effect [19.6 (12.6–53.7) mg;  $P>0.05$ ]. The thrombogenicity of the microparticles correlated strongly with their TF exposure ( $r=0.9524$ ,  $P=0.001$ ). *Conclusions:* Human cell-derived microparticles promote thrombus formation

*in vivo* in a TF-dependent manner. They might be the direct cause of an increased thromboembolic tendency in various patient groups.

**Keywords:** experimental animal model, microparticles, thrombus, tissue factor.

## Introduction

The primary initiator of coagulation *in vivo* is tissue factor (TF) [1]. According to the classical view, under normal conditions TF is not present intravascularly but is abundantly present in the vessel walls. Exposure of blood to TF upon vascular injury initiates normal hemostasis or, under pathological conditions such as disruption of an atherosclerotic plaque, thrombosis. This classical view has been challenged by *ex vivo* studies showing that potentially thrombogenic TF is also present in the circulating blood, even under normal conditions [2,3].

However, the source of TF circulating in blood has not been established. It is also not clear whether this TF circulates freely or whether it is associated with phospholipids or other structures. In the *ex vivo* thrombi formed on a surface devoid of TF, TF-positive membrane vesicles that also stained positive for the leukocyte marker  $\beta_2$ -integrin (CD18) were identified, as well as TF-positive neutrophil granulocytes and monocytes [2]. In addition, human atherosclerotic plaques contained high levels of TF-positive microparticles originating from apoptotic monocytes and lymphocytes [4]. Mechanical plaque disruption resulted in shedding of active TF associated with membranes, and intracoronary injection of human atherosclerotic plaque material in a porcine model caused microvascular thrombosis and coronary no-reflow [5]. Thus, leukocytes and disrupted atherosclerotic plaques may contribute to blood-borne, thrombogenic TF.

TF was recently found to be present on platelets, both resting and activated *in vitro*, and in the supernatant of these platelets [6–8]. TF in the supernatant was partly associated with

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microparticles released from the platelets [8]. Isolated circulating platelet-derived microparticles were also shown to contain TF, which was active in *in vitro* coagulation tests [9].

In a number of previous studies we identified cell-derived microparticles in the circulation of healthy individuals and patients suffering from certain diseases, especially sepsis. These microparticles can provide the anionic phospholipid surface necessary for coagulation and in some cases also expose TF. Thus, they can promote thrombin generation via TF/factor FVII-dependent and -independent pathways *in vitro* [10–13]. However, the thrombogenicity of microparticles isolated from human blood has not been investigated *in vivo*. For this purpose, we isolated microparticles from blood of healthy individuals and from blood obtained from the pericardial cavity of patients undergoing cardiac surgery with cardiopulmonary bypass (CPB), and tested their *in vivo* thrombogenicity in a rat model. Microparticles from healthy individuals were chosen because they initiate coagulation *in vitro* independent of TF [10], and pericardial microparticles because they initiate coagulation *in vitro* via TF [11].

## Methods

### Healthy individuals

After informed consent was obtained, venous blood from three healthy individuals [one woman, two men; age 47 (34–52) years] who had not taken any medication during the previous 10 days was collected into 0.1 volume of 105 mmol L<sup>-1</sup> trisodium citrate. Blood cells were removed by centrifugation (1550 g, 20 min, 20 °C), the plasma was snap-frozen in liquid nitrogen and stored at -80 °C. Pooled plasma from 20 healthy individuals was prepared similarly.

### Patients

This study was approved by the medical ethics committee of the Onze Lieve Vrouwe Gasthuis, and complies with the principles of the Declaration of Helsinki. Five patients [one female, four males, age 72 (65–76) years] undergoing elective coronary artery bypass grafting with CPB were included, after their informed consent. Patients with severe heart failure, renal or hepatic dysfunction, or a bleeding diathesis were excluded. Anesthesia, surgical procedure and treatment were described before [14]. Blood samples from the pericardial cavity were collected at the start of the last distal anastomosis, and processed as described above.

### Isolation of microparticles

Plasma samples (250 µL) were thawed on melting ice and pericardial samples were incubated with 2.5 µL heparinase for 15 min at room temperature to neutralize heparin received during cardiac surgery. The heparinase was prepared by dissolving Hepzyme (Dade Behring GmbH, Marburg, Germany) in 100 µL PBS (154 mmol L<sup>-1</sup> NaCl, 1.4 mmol L<sup>-1</sup> phosphate,

pH 7.4). In control experiments, 2.5 µL of this solution degraded at least 5 U mL<sup>-1</sup> unfractionated heparin, and itself did not affect microparticle-induced thrombin generation. Subsequently, samples were centrifuged at 17570 × g for 30 min at 20 °C, then 225 µL (227.5 µL for heparin-treated samples) of the essentially microparticle-free supernatant were removed. PBS containing 10.5 mmol L<sup>-1</sup> trisodium citrate was added (225 µL), microparticles were resuspended, centrifuged, and again 225 µL supernatant were removed. For flow cytometric analysis 175 µL PBS/citrate buffer, and for fibrin generation tests (FGTs) 75 µL PBS/citrate buffer were added to the remaining 25 µL, and the microparticles were resuspended. For *in vivo* experiments, 75 µL PBS/citrate buffer, or 37.5 µL antibody plus 37.5 µL PBS/citrate buffer, were added, the microparticles resuspended, and incubated for 30 min at room temperature, before injection into the animals. Antibodies used were antihuman TF (1 mg mL<sup>-1</sup>, product no. 4502; American Diagnostica Inc., Greenwich, CT, USA) and antihuman FXII (anti-hFXII), which did not inhibit contact activation-induced coagulation of rat plasma (this study) and therefore served as a control antibody (0.71 mg mL<sup>-1</sup>, OT-2, provided by the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, the Netherlands).

### Flow cytometric analysis

Analysis was performed by a modification of our previously published method [10,11]. Triple labeling of microparticles was performed in buffer consisting of PBS containing 2.5 mmol L<sup>-1</sup> CaCl<sub>2</sub> and 5% (v/v) defibrinated microparticle-free pooled human plasma (pH 7.4). This plasma was prepared by defibrinating pooled plasma from 20 healthy individuals with reptilase (Roche, Basel, Switzerland) as described previously [10,11], and centrifuging it at 22 000 × g for 60 min at 20 °C to remove remnants of fibrin and all microparticles.

Microparticle suspensions (5 µL) were diluted in 35 µL buffer. Annexin V-allophycocyanin (annexin V-APC; 5 µL) and a fluorescein isothiocyanate (FITC)- and a phycoerythrin (PE)-labeled monoclonal antibody (5 µL each) were added, or the respective isotype-matched control antibodies. The mixtures were incubated in the dark for 30 min at room temperature. Subsequently, 200 µL buffer were added and the suspensions were centrifuged at 17570 × g for 30 min at 20 °C. Finally, 200 µL of (microparticle-free) supernatant were removed, the microparticles were resuspended after addition of 300 µL buffer, and analyzed on a FACSCalibur flow cytometer with CELLQuest 3.1 software (Becton Dickinson and Co. Immunocytometry Systems, San Jose, CA, USA). Acquisition was performed for 1 min per sample, during which the flow cytometer analyzed 60 µL of the suspension. Forward scatter and side scatter were set at logarithmic gain. Microparticles were identified based on forward scatter, side scatter, and binding of annexin V. To identify annexin V-positive events, a threshold was placed based on a microparticle sample prepared with the use of calcium-free buffer. To identify cell marker and TF-positive events, thresholds were set based on microparticle

samples incubated with similar concentrations of isotype-matched control antibodies. Calculation of the number of microparticles per liter plasma was based upon the particle count per unit time, the flow rate of the flow cytometer, and the net dilution during sample preparation of the analyzed microparticle suspension. Microparticle-exposed TF antigen concentrations were calculated in each sample by multiplying the total concentration of TF-positive microparticles by the mean fluorescence intensity (MFI; arbitrary units) of the TF antigen exposure of the total TF-positive microparticle population.

IgG1-FITC, IgG1-PE (both clone X40) and CD14-PE (clone MφP9, IgG2b) were obtained from Becton Dickinson Immunocytometry Systems, IgG2b-PE (clone MCG2b) from Immuno Quality Products (Groningen, the Netherlands), CD61-PE (clone VI-PL2, IgG1) from PharMingen (San Jose, CA, USA), CD66e-PE (clone CLB-gran/10, IH4Fc, IgG1) from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, antiglycophorin A-PE (clone JC159, IgG1) from Dako (Glostrup, Denmark), anti-TF-FITC (product no. 4508CJ, IgG1) from American Diagnostica Inc., and annexin V-APC from Caltag Laboratories (Burlingame, CA, USA).

#### *Fibrin generation test*

As a source of clotting factors, pooled human plasma (prepared as described above) or pooled rat plasma was used. For preparation of rat plasma, blood was taken from the abdominal aorta of four rats anesthetized as described below, into 0.1 volume of 129 mmol L<sup>-1</sup> trisodium citrate. Blood cells were removed by centrifugation (2000 × g, 12 min, 20 °C), and the plasma was stored at -20 °C. For FGTs, the human and rat pool plasma were centrifuged (17570 × g, 30 min, 20 °C) to remove microparticles in them.

Microparticle suspensions or, as a negative control, saline (10 μL) were added to this human or rat plasma (75 μL), then either 15 μL buffer A (50 mmol L<sup>-1</sup> Tris-HCl, 100 mmol L<sup>-1</sup> NaCl, pH 7.35), antihuman TF, or antihuman FXII were added. After incubation for 30 min at room temperature, clotting was initiated by addition of CaCl<sub>2</sub> (15 μL; 100 mmol L<sup>-1</sup>). Fibrin formation was monitored by kinetic measurement of the optical density at λ=405 nm (SPECTRAMax microplate reader; Molecular Devices Corp., Sunnyvale, CA, USA). The time elapsed until the beginning of fibrin formation (lag time) was determined by extrapolation to the base line.

To test the specificity of the antibodies, FGTs were performed with various preparations instead of the microparticles: (i) rat thromboplastin prepared as described by Quick [15] (0.08 g L<sup>-1</sup> dried brain extract in saline); (ii) human placental thromboplastin (Thromborel S; Behring Diagnostics GmbH) diluted 100-fold with saline; (iii) a mixture of 8 μL kaolin (25 mg L<sup>-1</sup>; B.L.B. Laboratoires du Bois de Boulogne, Puteaux, France) and 2 μL phospholipid vesicles prepared according to Brunner [16] [185 μmol phospholipid L<sup>-1</sup>; 20% phosphatidylserine, 80% phosphatidylcholine (P7769 and L4129; Sigma, St Louis, MO, USA)].

#### *Venous stasis thrombosis model*

Male Wistar Hsd/Cpb; WU rats (*n* = 38, body weight 300–350 g) were obtained from Harlan (Horst, the Netherlands). All procedures were approved by the Ethics Committee of Animal Welfare of Organon in accordance with Dutch guidelines. Thrombus formation was studied as described previously [17]. Rats were anesthetized by intraperitoneal injection of 60 mg kg<sup>-1</sup> pentobarbital (Nembutal; Sanofi, Toulouse, France). The abdomen was opened and the vena cava inferior isolated. All side branches distal to the left renal vein were obliterated. Afterwards, 100 μL of rabbit thromboplastin suspension or saline (as positive and negative controls, respectively), or the 100-μL suspension of microparticles prepared as described above, were injected into the dorsal penile vein. The thromboplastin suspension was prepared by diluting Simplastin (Organon Teknika Corp., Durham, NC, USA) 50-fold with saline. After injection, blood was allowed to circulate freely for 10 s (total body blood circulation time in the rat is approximately 5 s), then the vena cava was ligated beneath the left renal vein and the abdominal cavity provisionally closed. After stasis was maintained for 10 min, the abdominal cavity was reopened, the vena cava was ligated near the fusion of the iliac veins and then opened longitudinally. The formed thrombus was removed and its wet weight determined.

Thromboplastin was injected into four rats, saline also into four. Each patient sample was injected into four rats: into two rats without antibodies, one rat after preincubation of microparticles with antihuman TF, and one rat after preincubation with anti-FXII. Samples from the three healthy individuals were injected into six, two, and two rats, respectively.

#### *Statistical analysis*

Data were analyzed with SPSS for Windows 9.0.0 (SPSS Inc., Chicago, IL, USA) and GraphPad PRISM 3.02 (GraphPad Software, Inc., San Diego, CA, USA). Differences between groups were analyzed with the Mann-Whitney *U*-test in case of two groups, and the Kruskal-Wallis test followed by Dunn's post test in case of more than two groups, to account for multiple comparisons. Correlations were determined with Spearman's rank correlation test. The logistic dose-response curve was obtained by fitting the equation  $y = y_{\min} + (y_{\max} - y_{\min}) / [1 + (x/EC_{50})^{-n}]$  to the data. In the equation, *x* represents microparticle-exposed TF antigen levels, *y* represents thrombus weight, EC<sub>50</sub> the half-maximal TF antigen level, and *n* the Hill coefficient. Differences and correlations were considered significant at *P* < 0.05. Data are presented as median with range, unless indicated otherwise.

## **Results**

#### *Numbers and cellular origin of microparticles*

The total number of microparticles (defined based on forward scatter/side scatter characteristics and annexin V binding) did

**Table 1** Numbers, cellular origin, and TF exposure of microparticles in pericardial plasma and in plasma of healthy individuals

	Pericardial plasma (n = 5)	Plasma of healthy individuals (n = 3)	P
Total number of MP ( $\times 10^6 L^{-1}$ plasma)	8982 (2214–12230)	6628 (5787–9274)	ns
Platelet-derived MP (CD61; % of total MP)	46 (25–55)	95 (95–96)	*
Erythrocyte-derived MP (GPA; % of total MP)	43 (32–62)	6 (4–17)	*
Granulocyte-derived MP (CD66e; % of total MP)	10 (8–24)	2 (1–3)	*
TF exposure of platelet-derived MP (TF+CD61; % of CD61-positive MP)	22 (11–26)	4 (3–10)	*
TF exposure of erythrocyte-derived MP (TF+GPA; % of GPA-positive MP)	21 (11–25)	17 (14–18)	ns
TF exposure of granulocyte-derived MP (TF+CD66e; % of CD66e-positive MP)	31 (23–47)	51 (41–64)	ns
Total number of TF exposing MP ( $\times 10^6 L^{-1}$ plasma)	1023 (299–1274)	347 (244–629)	ns
MP-exposed TF antigen (arbitrary units $L^{-1}$ plasma)†	65661 (60829–116152)	32366 (15898–49196)	*

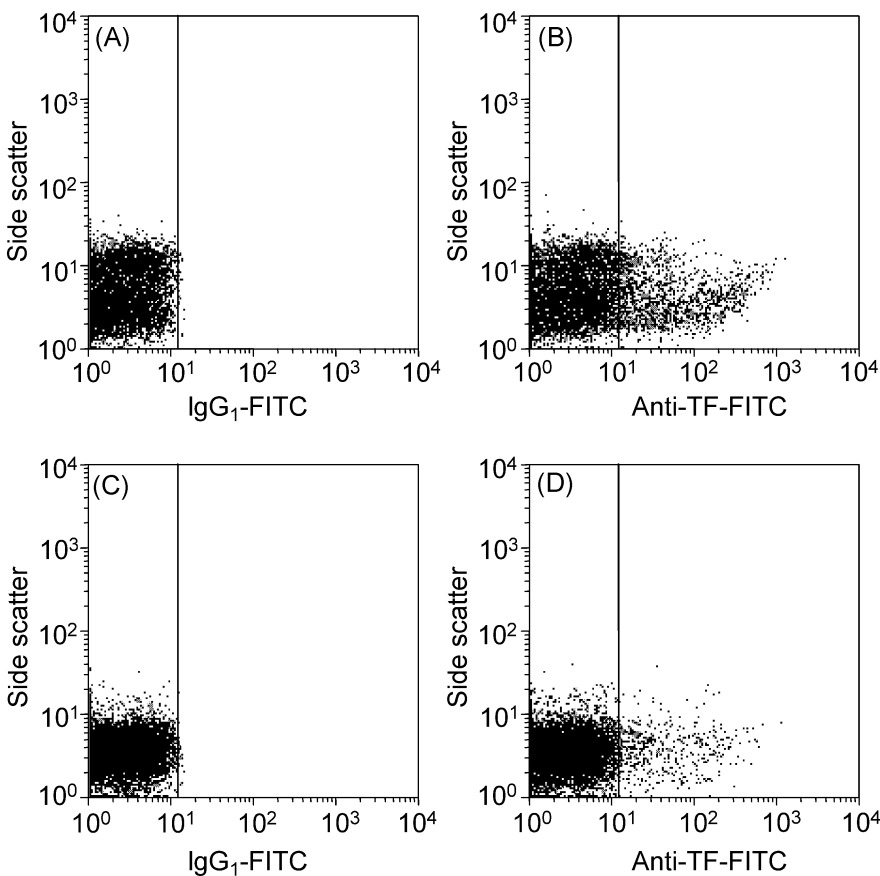
Values are given as median with range. Differences between pericardial samples and samples from healthy individuals were analyzed with the Mann–Whitney *U*-test. Two-tailed significance levels are provided (*P*), which were considered significant when  $P < 0.05$  (indicated by \*). ns, Non-significant differences; MP, microparticles; GPA, glycoprotein A. †MP-exposed TF antigen concentrations were calculated as described in Methods.

not differ significantly between pericardial samples and samples from healthy individuals ( $P = 0.786$ ; Table 1). This was due to the fact that pericardial plasma samples were 8–10-fold diluted during surgery with cardioplegic solution and saline. However, regarding their cellular source, the microparticles showed a different distribution in the two groups (Table 1). In samples from healthy individuals, most microparticles originated from platelets, with erythrocyte- and granulocyte-derived microparticles constituting a minor fraction. In contrast, pericardial samples contained erythrocyte-derived microparticles at

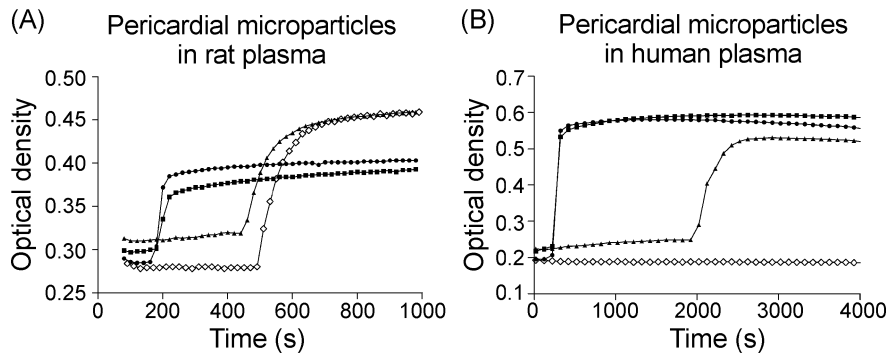
just as high numbers as platelet-derived microparticles. Also, the percentages of granulocyte-derived microparticles were higher in patient samples vs. samples from healthy individuals. Virtually no monocyte-derived microparticles were present in either group of samples.

#### TF exposure of microparticles

Microparticles from platelets, erythrocytes, as well as granulocytes stained positively for TF (Table 1). In pericardial samples,



**Fig. 1.** Representative flow cytometry dot plots of microparticles with respect to tissue factor (TF) exposure. Microparticles isolated from pericardial plasma of a patient undergoing cardiac surgery with cardiopulmonary bypass (CPB) (A,B) and microparticles isolated from plasma of a healthy individual (C,D) are shown. The dot plots were gated based on forward scatter/side scatter characteristics and annexin V binding. Anti-TF-FITC fluorescence vs. side scatter is shown (B,D), with fluorescence thresholds set using isotype-matched control antibodies (A,C).



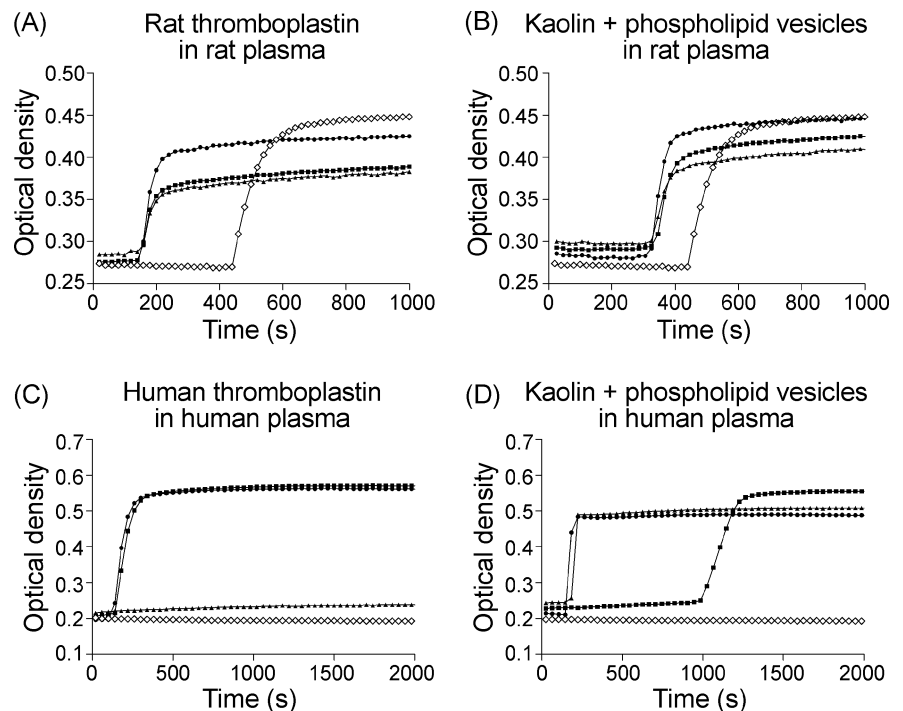
**Fig. 2.** Human microparticles induce clotting of rat plasma *in vitro*. (A) Microparticles were isolated from blood obtained from the pericardial cavity of patients undergoing cardiac surgery with cardiopulmonary bypass (CPB), and were tested in a fibrin generation test (FGT) in rat plasma without antibodies (●), or after preincubation with antihuman tissue factor (TF) (▲) or antihuman factor XII (■). A representative example is shown. (B) For comparison, the same experiment was performed in human plasma. In A and B, saline was used (instead of the microparticles) as negative control (◇).

the percentage of platelet-derived microparticles exposing TF was much higher than in samples from healthy individuals ( $P=0.036$ ). The percentage of erythrocyte- and granulocyte-derived microparticles exposing TF did not differ between the two groups ( $P=0.250$  and  $P=0.071$ , respectively).

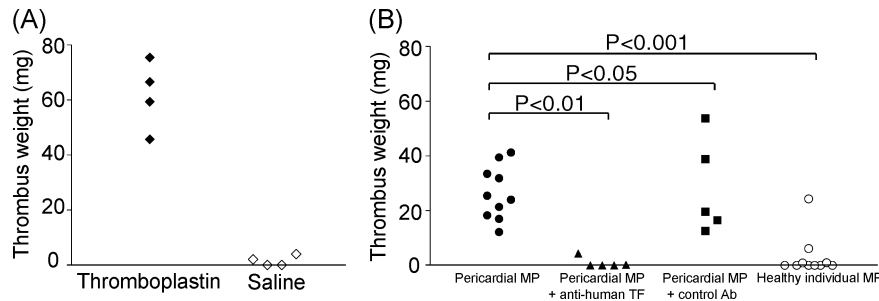
The total numbers of TF-exposing microparticles (Table 1) tended to be higher in the patient samples compared with samples from healthy individuals; this difference was nonetheless statistically not significant ( $P=0.143$ ). Representative flow cytometry dot plots are shown in Fig. 1. Microparticle-exposed TF antigen concentrations in the samples were calculated by multiplying the total numbers of TF-positive microparticles by their MFI as determined by flow cytometry. Pericardial samples had higher levels of microparticle-exposed TF compared with samples from healthy individuals ( $P=0.036$ ; Table 1).

#### *In vitro fibrin generation tests in rat plasma*

Without microparticles, rat plasma clotted after a lag time of 490 s. Pericardial microparticles shortened this lag time to 175 s (173–185 s) (median and range). Anti-human TF abolished this shortening [455 s (445–488 s)], while anti-hFXII had no effect [185 s (180–202 s)]. Microparticles from plasma of healthy individuals only minimally shortened the lag time [467 s (425–476 s)]. Figure 2A shows a representative FGT with human pericardial microparticles in rat plasma. Similar results were obtained in human instead of rat plasma (Fig. 2B). However, because the centrifugation procedure that removes all thrombin-generating microparticles from human plasma was insufficient to remove microparticles completely from rat plasma, the rat plasma eventually clotted even in the absence of added microparticles.



**Fig. 3.** The antihuman tissue factor (TF) and antihuman factor XII antibodies do not cross-react with the rat proteins. In a fibrin generation test (FGT), clotting was induced in rat plasma with rat thromboplastin (A), or via contact activation with kaolin plus artificially prepared phospholipid vesicles (B). In human plasma, clotting was induced with human thromboplastin (C), or via contact activation (D). In A–D, saline was used as negative control (◇). Experiments were performed without (●), or after preincubation with antihuman TF (▲) or antihuman factor XII (■).



**Fig. 4.** Thrombogenic activity of human cell-derived microparticles *in vivo* in rats. (A) Thrombus weights obtained after injection of thromboplastin (◆; four rats) or saline (◇; four rats). (B) Thrombus weights obtained after injection of pericardial microparticles (MP) without (●; five patient samples injected into two rats each), or after preincubation with antihuman TF (▲; five patient samples injected into one rat each) or antihuman factor XII as a control antibody (control Ab; □ five patient samples injected into one rat each), and thrombus weights obtained after injection of microparticles from plasma of healthy individuals (○; three healthy individual samples injected into six, two, and two rats, respectively). Microparticles injected were in every case isolated from 250  $\mu$ L of plasma, and brought to a final volume of 100  $\mu$ L with or without inhibitory antibodies. Each data point in the figure represents the thrombus weight obtained in one rat.

### Specificity of antibodies

To determine whether the antihuman TF and antihuman FXII antibodies cross-reacted with the respective rat proteins, we performed FGTs in rat plasma with clotting initiated by rat thromboplastin, i.e. a preparation containing TF and phospholipids from rat brain (Fig. 3A), or via contact activation using a mixture of kaolin and artificially prepared phospholipid vesicles (Fig. 3B). In either case, the antibodies had no effect. On the other hand, the same experiments performed in human plasma showed that antihuman TF specifically inhibited clotting induced by human thromboplastin (Fig. 3C), and anti-hFXII specifically inhibited contact activation of human coagulation factors (Fig. 3D). Thus, the antibodies reacted only with the respective human proteins and not with those of the rat.

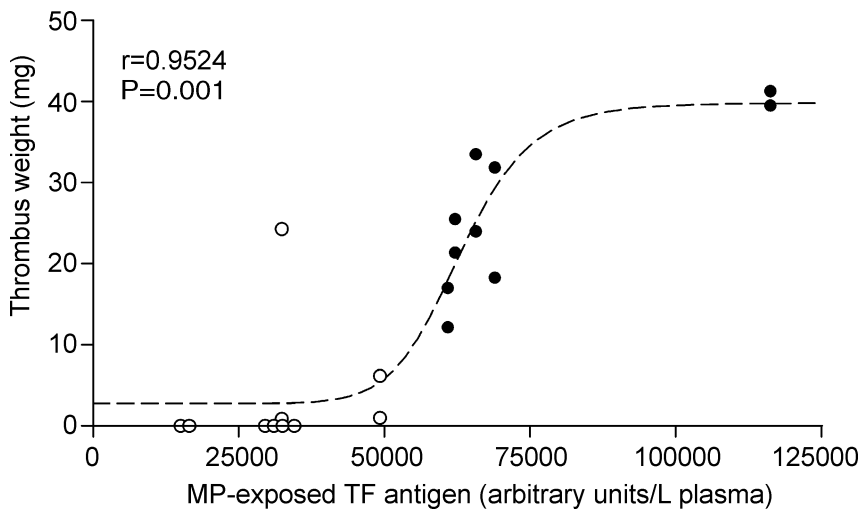
### Thrombogenicity of human microparticles in a rat model

The *in vivo* thrombogenic effect of the microparticles was examined using a venous stasis thrombosis model. As a positive control, thromboplastin was injected into the rats at a concen-

tration known to induce maximal thrombus weight [17]. This resulted in thrombi of 63 mg (45.7–75.5 mg). Saline served as negative control, and gave 1.1 mg (0–4 mg) thrombi (Fig. 4A).

Pericardial microparticles were highly thrombogenic, inducing thrombi of 24.8 mg (12.2–41.3 mg), whereas microparticles from plasma of healthy individuals were not, resulting in thrombi of 0 mg (0–24.3 mg;  $P < 0.001$  compared with pericardial samples). The thrombus weight of 24.3 mg was an outlier obtained in one of six rats receiving a microparticle suspension from the same healthy individual. The other five values were 0 mg in each of four rats, and 0.9 mg in one rat. Preincubation of pericardial microparticles with antihuman TF abolished their thrombogenic effect [0 mg (0–4.4 mg),  $P < 0.01$ ], while preincubation with anti-hFXII (which only functioned as a control antibody, given the fact that it did not inhibit contact activation of rat coagulation factors) had no effect [19.6 mg (12.6–53.7 mg),  $P > 0.05$ ] (Fig. 4B).

The microparticle-exposed TF antigen levels in the individual samples correlated highly with the *in vivo* thrombogenicity of the samples, i.e. the thrombus weights obtained in the rat model ( $r = 0.9524$ ,  $P = 0.001$ ; Fig. 5).



**Fig. 5.** Microparticle (MP)-exposed tissue factor (TF) antigen levels correlate strongly with the thrombogenicity of the microparticle samples. Microparticle-exposed TF antigen levels in the pericardial samples (●) as well as in samples from healthy individuals (○) were plotted against the thrombus weights obtained with the respective samples in *in vivo* experiments. Each data point represents the thrombus weight obtained in one rat. Spearman's rank correlation test was performed ( $r$ , correlation coefficient;  $P$ , significance level). Also, a logistic dose–response curve was fitted to the data (dotted line,  $r = 0.9770$ ,  $P < 0.01$ ).

## Discussion

In this study we demonstrated that microparticles promote thrombus formation *in vivo* in a TF-dependent manner. In meningococcal sepsis with disseminated intravascular coagulation, we previously detected significant numbers of monocyte-derived microparticles, exposing substantial amounts of TF [12]. Extrapolating the results of the present study leads us to conclude that those microparticles probably contribute to the severe clotting abnormalities seen in meningococcal sepsis. In the present study, however, virtually no monocyte-derived microparticles (CD14) were found, ruling out monocytes as an important source of microparticles under normal conditions as well as in the cardiac surgery patients. Practically all microparticles could be identified using the cell markers CD61 (platelets), glycophorin A (erythrocytes), and CD66e (granulocytes).

Microparticles derived from granulocytes, platelets, as well as erythrocytes stained positively for TF. *De novo* synthesis of TF under our study conditions was unlikely, considering the duration of the surgical intervention. TF-positive neutrophil granulocytes have repeatedly been observed in circulating blood as well as in *ex vivo* thrombi [2,18] under conditions excluding *de novo* protein synthesis, so it seems quite feasible that microparticles released from these cells would also expose TF. Our findings of TF-positive platelet-derived microparticles are in line with those of other groups [6–9]. Platelets can acquire TF from monocytes and neutrophils via microparticles [18], and the reverse process has also been shown [8]. The mechanism by which erythrocyte-derived microparticles could acquire TF is unknown.

We used flow cytometry to give an indication of the TF antigen exposed on both pericardial and healthy individual microparticles, given the fact that the sensitivity of the ELISA is too low for the latter (and more concentrated microparticle preparations give unreliable results due to the matrix effect of adding so much lipid). Also, the commercially available ELISAs contain a detergent in the sample buffer and therefore do not necessarily measure only surface-exposed TF but also TF within the microparticles, which as such is unlikely to contribute to the TF activity. In another study we established using an ELISA that pericardial plasma, diluted about 10 times with cardioplegic solution and saline during surgery, contains in total  $122 \text{ ng L}^{-1}$  TF (median), with  $85 \text{ ng L}^{-1}$  (72%; median) of this TF associated with microparticles [19]. In plasma of healthy individuals, we found total TF concentrations of  $80\text{--}140 \text{ ng L}^{-1}$  and the amount of TF associated with the microparticles was below the detection limit of the ELISA (unpublished data). Given a lower detection limit of  $10 \text{ ng L}^{-1}$ , pericardial samples contain at least 8-fold higher TF quantities associated with microparticles. However, the median surface exposure—and thus potentially active fraction—is only twice higher (present study, Table 1).

We found a very high correlation between the measured total microparticle-exposed TF antigen and the thrombus weights obtained by intravenous injection of these microparticles in a rat thrombosis model. A possible explanation for the thrombogenicity of microparticle-exposed TF might be the threshold phenomenon (Fig. 5, dotted line), i.e. above a certain level microparticle-exposed TF would be able to overcome inhibitors of coagulation and initiate thrombus formation. However, it is also possible that the low thrombogenicity of microparticles of healthy individuals results not from the fact that the TF exposed on them is below a certain threshold, but from encryption of TF, i.e. an inactive state of the TF antigen on these microparticles. Potential mechanisms of TF encryption on cells are known to include effects of the lipid microenvironment within the membrane [20], association of TF with caveolae [21,22], and changes in the quaternary structure of TF [23], but have not been studied on microparticles so far. Possible differences in phospholipid composition between microparticles of healthy individuals and cardiac surgery patients may also influence the prothrombotic potential of the microparticles [24,25] independently of any effect on TF encryption. Furthermore, since we used no inhibitors of rat contact activation, the role of this process in the thrombogenic effect of the microparticles cannot be excluded. However, since antihuman TF practically abolished thrombus formation, the role of contact activation cannot be prominent.

Several conditions with a thromboembolic tendency have been shown to be associated with increased numbers of circulating microparticles, e.g. heparin-induced thrombocytopenia [26], patients with lupus anticoagulant [27], cerebrovascular accidents [28], acute coronary syndromes [29,30], or patients undergoing cardiac surgery with CPB [31]. These microparticles were shown to be procoagulant by virtue of their phospholipid surface. On the other hand, elevated plasma levels of TF were also measured in such conditions, e.g. disseminated intravascular coagulation [32,33], acute myocardial infarction [34], unstable angina pectoris [35,36], the antiphospholipid syndrome [37], or patients undergoing cardiac surgery with CPB [38]. It is likely that the plasma TF that is elevated in these conditions is at least partially microparticle-bound, and the increased numbers of microparticles found in the circulation of these patients thus not only provide the anionic phospholipids necessary for coagulation but may also expose active TF on their surface. Direct *in vivo* evidence linking circulating microparticles to thromboembolic complications has, however, hitherto been missing. In this study we showed for the first time that human cell-derived microparticles promote thrombus formation *in vivo* in a TF-dependent manner. They might be the direct cause of an increased thromboembolic tendency in various patient groups.

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