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REGULAR ARTICLE

Phospholipid composition of *in vitro* endothelial microparticles and their *in vivo* thrombogenic properties

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Abstract

Introduction: Microparticles from activated endothelial cells (EMP) are well known to expose tissue factor (TF) and initiate coagulation *in vitro*. TF coagulant activity is critically dependent on the presence of aminophospholipids, such as phosphatidylserine (PS) and phosphatidylethanolamine (PE), but it is unknown whether or not TF-exposing EMP are enriched in such aminophospholipids. Furthermore, despite the fact that EMP have been reported in several pathological conditions, direct evidence for their (putative) coagulant properties *in vivo* is still lacking. We investigated the phospholipid composition of endothelial MP (EMP) and their thrombogenic properties *in vivo*.

Materials and methods: Human umbilical vein endothelial cells (HUVEC; $n=3$) were incubated with or without interleukin (IL)-1α (5 ng/mL; 0–72 h). Phospholipid composition of EMP was determined by high-performance thin layer chromatography. The association between EMP, TF antigen and activity was confirmed *in vitro* (ELISA, Western blot and thrombin generation). Thrombogenic activity of EMP *in vivo* was determined in a rat venous stasis model.

Results: Levels of TF antigen increased 3-fold in culture medium of IL-1α-treated cells ($P<0.0001$). This TF antigen was associated with EMP and appeared as a 45–47 kDa protein on Western blot. In addition, EMP from activated cells were enriched in both PS ($P<0.0001$) and PE ($P<0.0001$), and triggered TF-dependent thrombin formation *in vitro*

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and thrombus formation *in vivo*. In contrast, EMP from control cells neither initiated coagulation *in vitro* nor thrombus formation *in vivo*.
Conclusions: EMP from activated endothelial cells expose coagulant tissue factor and are enriched in its cofactors PS and PE.

Introduction

Tissue factor (TF), a 45–47 kDa transmembrane receptor, initiates coagulation [1], triggers cell migration [2] and trafficking of mononuclear phagocytes across the endothelium [3], regulates angiogenic properties of tumor cells [4], acts as a chemotactic factor for vascular smooth muscle cells [5], and protects endothelial cells from apoptosis [6,7]. TF is widely distributed within the body. Extravascular cell types constitutively express TF [8,9], and cells at the blood interface (endothelial cells) or circulating within the blood (monocytes) inducibly express TF [10–13].

TF can also be present on cell-derived microparticles (MP) *in vivo*. MP isolated from pericardial wound blood [14], synovial fluid [15] or venous blood from a patient with meningococcal septic shock complicated by fulminant disseminated intravascular coagulation [16] initiate TF-dependent thrombin generation *in vitro*. In addition, we demonstrated that MP from (pericardial) wound blood trigger TF-mediated thrombus formation *in vivo* [17]. As yet, other MP have not been demonstrated to have such activity *in vivo*.

Endothelial cell-derived MP (EMP) from TNFα-or LPS-activated endothelial cells expose procoagulant TF *in vitro* [18,19], but whether such EMP have any biological activity *in vivo* is unknown. This question is becoming increasingly relevant since elevated numbers of EMP are now known to occur in various pathological conditions, including systemic lupus erythematosus [20], thrombotic thrombocytopenia purpura [21], vasculitis of the young [22], paroxysmal nocturnal haemoglobinuria [23] and multiple sclerosis [24]. EMP in healthy subjects were reported to correlate with the serum triglyceride concentration, suggesting that EMP may reflect endothelial dysfunction or injury [25].

Aminophospholipids like phosphatidylserine (PS) and phosphatidylethanolamine (PE) are well established cofactors for the procoagulant activity of membrane-exposed TF [26–28]. Recently, we showed that the phospholipid composition of platelet-derived MP changes upon activation [29]. Whether or not the phospholipid composition of EMP changes during activation of endothelial cells, however, is unknown.

The aims of the present study were to study the presumed procoagulant properties of EMP *in vivo* and to determine whether phospholipid composition changes during endothelial cell activation may support this TF activity.

Materials and methods

Reagents and assays

Medium M199, penicillin, streptomycin, amphotericin B and L-glutamine were obtained from GibcoBRL, Life Technologies (Paisley, Scotland). IgG_1-FITC and IgG_1-PE (clone ×40) were obtained from Becton Dickenson ((BD) San Jose, CA). Annexin V-(allophycocyanin; APC) was from Caltag Laboratories (Burlingame, CA). Human serum albumin (HSA) and monoclonal antibodies (MoAbs), directed against factor VIIa (VII-1 [1.46 mg/mL], VII-15 [0.53 mg/mL]) and anti-factor XII (OT-2 [0.71 mg/mL), were from Sanquin (Amsterdam, The Netherlands). Anti-TF for Western blotting (4503, clone TF9-10H10, IgG_1) and anti-TF for *in vivo* studies (4502, polyclonal IgG) were from American Diagnostica Inc. (Greenwich, CT). Anti-mouse IgG-horseradish peroxidase (HRP) conjugate was from Bio-Rad (Hercules, CA). Recombinant human interleukin-1α (IL-1α), human recombinant basic fibroblast growth factor and epidermal growth factor were from GibcoBRL (Gaithersburg, MD). Collagenase (type 1A) was from Sigma (St. Louis, MO), EDTA from Merck (Darmstadt, Germany), heparin (400 U/mL) from Bufa BV (Uitgeest, The Netherlands), and trypsin from Difco Laboratories (Detroit, MI). Human serum was provided by the Blood Bank Center of the Leiden University Medical Center (Leiden, The Netherlands) and was heat inactivated during 30 min at 56 °C (HuSi). Tissue culture flasks were from Greiner Labortechnik (Frickenhausen, Germany) and gelatin from Difco Laboratories (Sparks, MD). Reptilase was from Roche (Mannheim, Germany) and the chromogenic substrate Pefachrome TH-5114 from Pentapharm Ltd. (Basel, Switzerland). Heparinase (Hepzyme) was from Dade Behring GmbH (Marburg, Germany). Human brain thromboplastin was a gift from Prof. Dr. R. Bertina (Department of Haematology, Leiden University Medical Center, Leiden, The Netherlands). Pentobarbital sodium (Nembutal) was obtained from Sanofi (Toulouse, France). L-α-lysophosphatidylcholine (L-PC; 38–0104), sphingomyelin (SM; 56–1080), L-α-phosphatidylcholine (PC; 37-0106), L-α-PS (37-0160), L-α-phosphatidylinositol (PI; 37-0134) and L-α-PE (37-0126) were from Larodan (Malmö, Sweden), L-α-lysophosphatidylethanolamine (L-PE; L4754) and cholesterol (C8667) from Sigma (St. Louis, MO), and L-α-lysophosphatidylserine (L-PS; 850092P) from Avanti Polar Lipids Inc. (Alabaster, AL). Chloroform, ethylacetate, acetone, methanol, ethanol, dichloromethane, isopropanol and acetic acid (all HPLC grade) were from Merck (Darmstadt, Germany). All other chemicals were of analytical quality.

Isolation, culture and treatment of human umbilical vein endothelial cells (HUVEC)

HUVEC were collected from human umbilical cord veins and cultured as described previously [20].

Isolation of EMP

At the indicated activation time intervals, culture supernatants were collected and centrifuged (10 min at 180 ×*g* and 20 °C) to remove detached cells. Aliquots (250 μL each) of supernatants were frozen in liquid nitrogen and stored at −80 °C. Samples were thawed on melting ice for 1 h and centrifuged for 30 min (17,570 ×*g* and 20 °C) to pellet EMP. Then, 225 μL supernatant was removed and the EMP-enriched pellet was washed once with 225 μL PBS/10.9 mmol/L trisodium citrate (pH 7.4). Finally, EMP were resuspended in the remaining 25 μL.

Flow cytometric analysis

EMP were analyzed in a FACSCalibur flow cytometer (BD). Forward scatter (FSC) and side scatter (SSC) were set at logarithmic gain and EMP were identified and quantified by their FSC and SSC characteristics and binding of annexin V as described previously [20].

Western blotting

Culture supernatants (5 mL) were collected after 24 h of incubation without or with IL-1α. Detached cells were removed by centrifugation (10 min at 180 ×*g* and 20 °C). EMP were pelleted (1 h at 17,570 ×*g* and 20 °C) and washed once in PBS/citrate. The final pellet was resuspended in 24 μL PBS, to which 6 μL (5-fold concentrated) sample buffer was added (β-mercaptoethanol (12.5% v/v), bromophenol blue (0.025% v/v), glycerol (25% v/v), SDS (10% w/v) and Tris base (312.5 mM; pH 6.8)). Samples were heated before electrophoresis (5 min, 100°C). Proteins were separated on 10% polyacrylamide gel and transferred to a nitrocellulose membrane (Schleicher & Schuell; Dassel, Germany). Subsequently, blots were incubated (at room temperature) with blocking buffer (Tris-buffered saline-Tween (TBST); 10 mmol/L Tris–HCl, 150 mmol/L NaCl, 0.05% (v/v) Tween-20; pH 7.4), containing 5% (w/v) dry milk powder (Protifar; Nutricia, Vienna, Austria); 60 min), (mouse) anti-human-TF (1 μg/mL; 60 min) and (goat) anti-mouse IgG-HRP conjugate (1:3000; 45 min). Between the incubation steps, blots were washed three times with TBST for 5–10 min. All antibodies were diluted with blocking buffer. The bands were detected using an enhanced chemiluminescence kit (ECL; Amersham Biosciences; Buckinghamshire, UK) and exposed to Fuji Medical X-ray film.

TF ELISA

TF in conditioned media was determined by ELISA (American Diagnostica Inc.; Greenwich, CT).

Thrombin generation assay

The procoagulant properties of EMP *in vitro* were studied in a thrombin generation test (TGT) as described previously [30]. In a control experiment, we found no effect of freeze–thawing on the ability of microparticles to initiate thrombin generation (data not shown). The ability to inhibit TF-initiated coagulation of the anti-human factor VII used in this study is comparable to that of anti-human TF previously used in our thrombin generation assay [16,30].

Rat venous stasis model

Rats were anesthetized and subsequently the abdomen was opened and the vena cava inferior was isolated. All side branches distal to the left renal vein were obliterated. Afterwards, rabbit thromboplastin suspension or saline (as positive and negative control, respectively) or EMP were injected into the dorsal penile vein. Thromboplastin suspension was prepared by diluting Simplastin 50-fold (v/v) with saline. After injection, blood was allowed to circulate freely for 10 s. Then the vena cava was ligated beneath the left renal vein. After maintaining stasis for 10 min, the vena cava was ligated near the fusion of the iliac veins, and then opened longitudinally. The formed thrombus was removed and weighed [17,31]. Briefly, aliquots (250 μL each) of (cell-free) conditioned medium from both activated (IL-1α, 5 ng/mL, 48 h) or resting HUVEC were thawed on melting ice and incubated with heparinase to degrade heparin, an essential cofactor of Fibroblast Growth Factor for endothelial cell culture. EMP were isolated and washed in PBS/citrate by centrifugation (30 min at 17,570 ×*g* and 20 °C). Before injection, EMP were resuspended in 75 μL PBS/citrate buffer (pH 7.4) or 37.5 μL antibody plus 37.5 μL PBS/citrate buffer. Antibodies used were polyclonal rabbit anti-human TF and anti-human factor XII. Male Wistar Hsd/Cpb; WU rats (n=32, body weight 300–350 g) were obtained from Harlan (Horst, The Netherlands). All procedures were approved by the Ethics

Table 1 Phospholipid composition of EMP from unstimulated (−) or activated (+) endothelial cells

Phospholipid	IL-1α	Culture time (h)				
		3	12	24	48	72
L-PC	−	8±1	8±1	11±4	10	13±2
	+	8±1	6±2	9±3	7	9±3
SM	−	17±1	16±2	23±2	19	18±1
	+	17±1	14±1.0	21±3	17	17±1
PC	−	57±2	54±6	45±3	57	51±4
	+	56±1	51±1	40±2$^{\#}$	45	42±2$^{\#}$
PS*	−	4±3	7±1	4±1	2	6±1
	+	3±1	10±3$^{\#}$	8±3$^{\#}$	6	11±1$^{\#}$
PI	−	5±2	4±1	8±1	3	4±1
	+	7±0	4±0	9±1	3	3±0
PE*	−	9±2	11±6	10±3	7	7±1
	+	11±0	14±0	14±3$^{\#}$	16	16±1$^{\#}$

Data are expressed as % of total phospholipid (mol/mol). *P< 0.0001 (area under curve), $^{\#}P$<0.05 (individual time points). Activation with IL-1α did not affect the relative amounts of L-PC (P=0.400), SM (P=0.100), PC (P=0.100) or PI (P=0.700). Data are shown as mean ± SD (n=3–5) except for the 48 h time interval, since EMP from this collection point had been arbitrarily chosen to be used in the rat venous stasis model and therefore insufficient material was available for further analysis.

Committee of Animal Welfare of Organon in accordance with Dutch guidelines.

Phospholipid extraction and high-performance thin layer chromatography (hpTLC)

EMP were isolated from aliquots of cell-free culture supernatants (1 mL; $n=3$) as reported earlier [32]. Lipids were extracted and phospholipids were separated and quantified as described previously [29,33,34].

Statistics

To determine whether activation of endothelial cells significantly affected the overall numbers of EMP and TF antigen levels in conditioned medium in time, area under curves were calculated and differences were post-analyzed using (two-tailed) paired *t*-test (GraphPad Prism for Windows, release 3.02 (San Diego, CA)). In case of a significant difference, data per time interval were further analyzed by two-tailed paired *t*-test. For individual phospholipids, the overall differences in time (3–72 h) between EMP from unstimulated *versus* stimulated endothelial cells were determined by calculating the "area under curve" represented by the data shown in Table 1, followed by Mann–Whitney test (two-tailed; MedCalc). When a significant difference of the "area under curve" was found to be present, also paired *t*-tests were performed to determine at

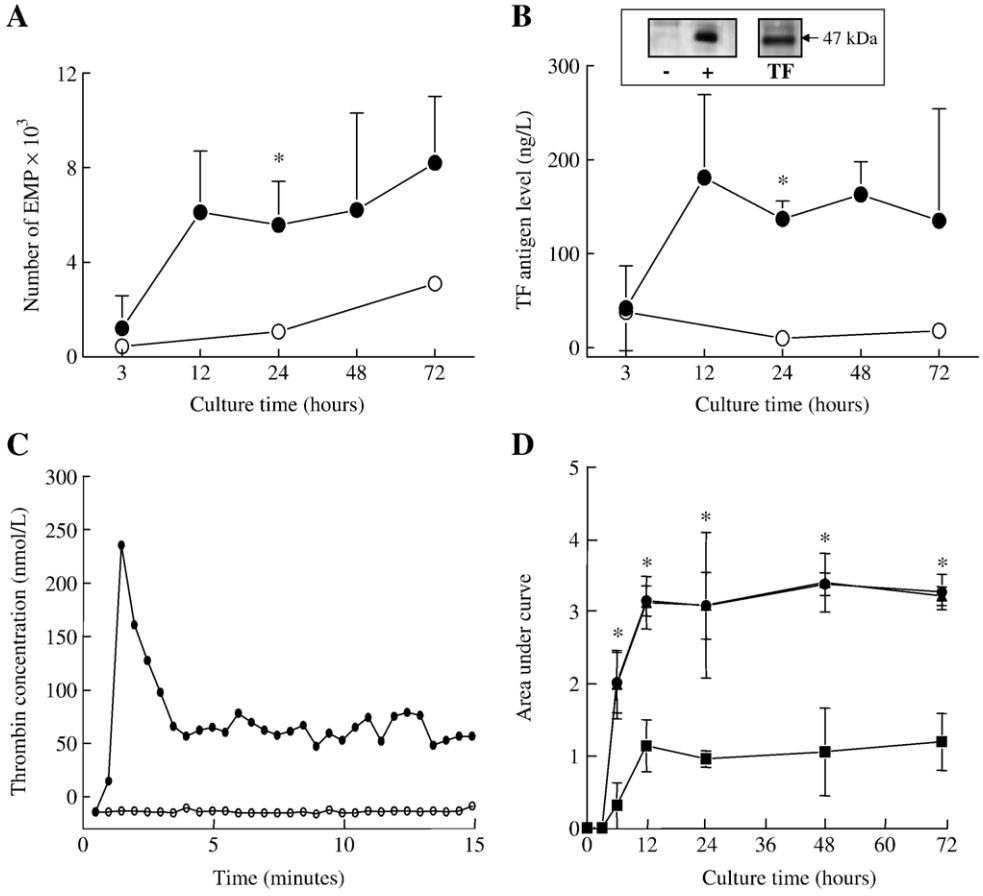

Figure 1 EMP from IL-1α-activated endothelial cells expose TF and are coagulant *in vitro* HUVEC were incubated with or without IL-1α (5 ng/mL; control samples were collected at 3 h, 24 h and 72 h; $n=3$). At the indicated time intervals conditioned media from control (○) and IL-1α-activated endothelial cells (●) were collected and analyzed. (A) Numbers of EMP identified by FSC, SSC and binding of annexin V. (B) TF antigen in conditioned medium containing the EMP (upon removal of the EMP, the conditioned medium did not contain detectable quantities of TF, indicating all TF to be EMP-associated); the insert shows a representative Western blot of EMP lysates from unstimulated (−) and activated (+) endothelial cells; human brain thromboplastin (TF) was used as a positive control. (C) EMP from unstimulated (○) and IL-1α-activated endothelial cells (●) were reconstituted in defibrinated, MP-free normal plasma to assess their thrombin generating capacity. Data from a representative thrombin generation experiment. (D) Thrombin generation without (●) or with anti-human factor VII (■) or anti-human factor XII (▲). The ability of EMP to generate thrombin was expressed as the area under the curve during 15 min of thrombin generation ($n=3$).*$P<0.05$ (EMP without antibody *versus* EMP incubated with anti-human factor VII).

which time points the differences were significant. Data from *in vitro* thrombin generation were analyzed by two-tailed paired *t*-test. Data on thrombus formation were analyzed using the Kruskal–Wallis test followed by Dunn's post test to correct for multiple comparisons. All differences were considered statistically significant at $P<0.05$. Values are expressed as mean ± SD.

Results

EMP from IL-1α-treated endothelial cells expose TF and trigger coagulation *in vitro*

After 72 h the numbers of EMP in conditioned medium from control (unstimulated) cultures had increased gradually about 6-fold compared to (conditioned medium from) 3 h control cultures (Fig. 1A). In contrast, upon activation with IL-1α, the numbers of EMP increased already about 13-fold after 12 h of culture compared to the 3 h time interval, and these numbers remained virtually constant up to 72 h of culturing. In IL-1α-treated cultures, the overall increase of EMP numbers in time differed significantly compared to control ($P=0.016$). For individual time intervals, a significant difference was observed at 24 h ($P=0.04$), but not at 3 h ($P=0.503$) or 72 h ($P=0.07$). Also, EMP numbers at the 48 h activation time interval were comparable to 24 or 72 h control conditions ($P=0.174$ and $P=0.324$; data not shown). Concurrently, the overall concentrations of the TF antigen in conditioned media of IL-1α-activated cells increased in time (Fig. 1B; $P=0.037$). This increase was significant at 24 h ($P=0.0024$), but not at 3 h ($P=0.931$) or 72 h ($P=0.241$). After removal of EMP by high-speed centrifugation, the concentrations of TF (antigen) in the supernatants were below the detection limit (12.5 ng/L), indicating that all non-endothelial cell-bound TF is associated with EMP (data not shown). The EMP-associated TF appeared as a single 45–47 kDa protein band on Western blot (insert Fig. 1B). Only EMP from activated endothelial cells initiated thrombin formation *in vitro* (Fig. 1C), and this was inhibited by anti-human factor VII(a) ($P=0.014$) but not by anti-human factor XII(a) (Fig. 1D; $P=0.896$). Both the total capacity of the EMP to generate thrombin and the factor VII-TF dependency remained unchanged between 12 and 72 h of culture. Thus, as shown previously for endotoxin or TNF-treated endothelial cells, also IL-1α-treated endothelial cells release TF-exposing EMP, which triggers (TF-dependent) thrombin generation *in vitro*.

Thrombus formation by EMP *in vivo*

In vivo, injection of Simplastin (positive control; a commercially available mixture of TF and lipids from rabbit brain tissues (Organon Teknika Corp.; Durham, NC)) gave thrombi of 61.8 mg ± 12.6 ($n=4$). Injection of saline (negative control) gave thrombi of 1.5 mg ± 1.9 ($n=4$; data not shown). Upon injection of EMP from activated endothelial cells, thrombi were formed (Fig. 2; 35.1 mg ± 12.9, $P<0.01$). Preincubation with anti-human TF significantly blocked thrombus formation (5.6 mg ± 9.3, $P<0.05$). In contrast, preincubation with anti-factor XII had less effect (26.9 mg ± 9.2, $P>0.05$) and was not statistically significant. In line with our *in vitro* observation, no thrombus formation was observed in rats that received EMP from unstimulated endothelial cells (0.5 mg ± 0.7). These data show that only EMP from activated human endothelial cells are strongly thrombogenic *in vivo* in a TF-dependent manner.

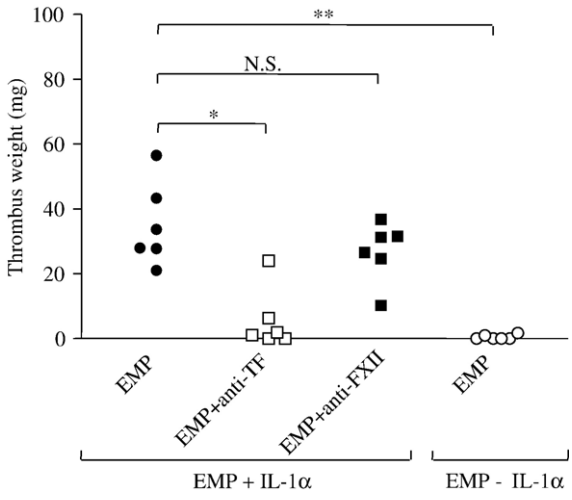

Figure 2 Thrombus formation by EMP *in vivo* EMP from unstimulated and IL-1α-activated endothelial cells (48 h) were injected into rats to assess their ability to trigger thrombus formation *in vivo*. EMP fractions from three different endothelial cell cultures were used. From each individual culture, EMP from unstimulated endothelial cells were injected into 2 rats (EMP- IL-1α). From the corresponding EMP of activated cells, EMP not preincubated with antibodies (EMP + IL-1α) were injected into 2 rats, EMP preincubated with anti-human TF (EMP + anti-TF) in 2 rats, and EMP preincubated with anti-human factor XII (EMP + anti-FXII) also in 2 rats. Thrombus weights for individual rats are indicated. N.S. (not significant; $P>0.05$); *$P<0.05$;**$P<0.01$.

Phospholipid composition of EMP

The most prominent phospholipids in EMP from both unstimulated and stimulated endothelial cells were PC and SM (Table 1). EMP from activated endothelial cells contained significantly increased amounts of both PS ($P<0.0001$) and PE (both $P<0.0001$) as compared to EMP from control cells. Upon activation of endothelial cells, the total amount of phospholipids in isolated EMP fractions tended to increase, although this increase did not reach significance ($P=0.2$). Similarly, the cholesterol:phospholipid ratio of EMP was unchanged upon activation ($P=0.4$).

Discussion

Previous studies demonstrated that EMP from TNF-α or LPS-treated endothelial cells expose TF and trigger thrombin generation *in vitro* [18,19]. Similarly, our present data show that also EMP from IL-1α-activated endothelial cells expose TF and trigger thrombin generation *in vitro*. More interestingly, however, is that such EMP become enriched in both PS and PE, and trigger thrombus formation *in vivo* by a TF-initiated pathway.

In this study we present data that TF exposed by EMP from activated endothelial cells is responsible for the coagulant activity *in vitro* and *in vivo*. This is

based upon control studies with EMP from non-activated HUVEC and from inhibitory studies with antibodies against the extrinsic pathway. It could be argued that the absence of a coagulant effect of EMP from the control situation is due to the fact that 2 to 3-fold lower numbers of EMP are present in the culture medium, *i.e.* a lower availability of procoagulant phospholipids. In our experiments we did not correct for that difference by taking larger volumes of medium, because the EMP numbers vary somewhat between experiments. However, with the EMP from the activated HUVEC, *i.e.* EMP exposing TF, inhibition of the extrinsic coagulation pathway completely abolished their ability to initiate coagulation at the same EMP concentration. Evidently, the exposure of procoagulant phospholipids is insufficient to trigger coagulation, although it may promote the TF-associated coagulant activity and facilitate the binding of coagulation factors.

Jimenez et al. studied the numbers and antigenic phenotype of EMP from microvascular- and macrovascular endothelial cells after activation (TFN-α) or induction of apoptosis (serum deprivation) [35]. They showed that EMP from microvascular- and macrovascular endothelial cells differed in antigenic composition. Moreover, they showed that the antigenic composition of EMP from both microvascular as well as macrovascular EMP was differentially affected upon activation or induction of apoptosis. For instance, whereas the numbers of annexin V-binding EMP, *i.e.* EMP exposing PS on their surface, from microvascular endothelial cells was increased during growth factor deprivation compared to EMP from activated (microvascular) endothelial cells, culture supernatants from macrovascular endothelial cells hardly contained any annexin V-binding EMP, *i.e.* not even when these cells had been subjected to growth factor deprivation resulting in apoptosis. In the present study, we used a different kind of endothelial cell (HUVEC), we used a different inducer to activate (IL-1α), and determined the antigenic composition of EMP after freeze–thawing. Therefore, the antigenic composition of EMP in these two studies, including the binding of annexin V, can not be directly compared. As for the PS exposure, we used EMP after snap freezing in liquid nitrogen, storage at $-20\ ^{\circ}C$ and subsequent thawing, which increases exposure of PS on the EMP. Thus, the EMP used in our present study can promote the coagulation process by enabling the formation of prothrombinase- and tenase complexes on their surface, but the presence of TF is necessary to initiate the coagulation cascade.

Recently, del Conde et al. showed that monocyte-derived MP may fuse with activated platelets, thereby transferring their TF [36]. It was suggested that MP predestined for fusion are likely to be enriched in fusion-promoting phospholipids like PS. Our present data confirm their hypothesis for EMP. Thus, differences in phospholipid composition (of MP) may not only affect their procoagulant properties but also their ability to deliver TF to target cells. The changes in phospholipid composition are likely to be cell-type and/or agonist dependent. Previously, we showed that upon platelet activation, the PS content of platelet-derived MP (PMP) was unaffected, whereas their cholesterol and sphingomyelin content increased [29].

Disseminated intravascular coagulation is a frequent complication of endotoxic shock. Drake et al. demonstrated systemic fibrin deposition in a lethal *Escherichia coli* sepsis baboon model. They failed, however, to demonstrate a significant occurrence of TF on endothelial cells [37]. They concluded, that "compared with endothelial cells in culture, there is *in vivo* significantly greater control of TF expression than expected". Our present data suggest that the absence of TF on endothelial cells can be explained by the release of TF-exposing EMP from these cells into the circulation. This explains on the one hand the systemic fibrin deposition and on the other hand the unexpected absence of TF on the endothelium *in vivo*.

Taken together, the present findings demonstrate that TF-exposing EMP are enriched in aminophospholipids, and that such EMP are highly thrombogenic *in vivo*.

References

[1] Nemerson Y. Tissue factor and hemostasis. *Blood* 1988;**71:** 1–8.
[2] Ruf W, Mueller BM. Tissue factor signaling. *Thromb Haemost* 1999;**82:**175–82.
[3] Randolph GJ, Luther T, Albrecht S, Magdolen V, Muller WA. Role of tissue factor in adhesion of mononuclear phagocytes to and trafficking through endothelium in vitro. *Blood* 1998; **92:**4167–77.
[4] Zhang Y, Deng Y, Luther T, Muller M, Ziegler R, Waldherr R, et al. Tissue factor controls the balance of angiogenic and antiangiogenic properties of tumor cells in mice. *J Clin Invest* 1994;**94:**1320–7.
[5] Sato Y, Asada Y, Marutsuka K, Hatakeyama K, Sumiyoshi A. Tissue factor induces migration of cultured aortic smooth muscle cells. *Thromb Haemost* 1996;**75:**389–92.
[6] Sorensen BB, Rao LV, Tornehave D, Gammeltoft S, Petersen LC. Antiapoptotic effect of coagulation factor VIIa. *Blood* 2003;**102:**1708–15.
[7] Versteeg HH, Spek CA, Richel DJ, Peppelenbosch MP. Coagulation factors VIIa and Xa inhibit apoptosis and anoikis. *Oncogene* 2004;**23:**410–7.
[8] Tedgui A, Mallat Z. Smooth muscle cells: another source of tissue factor-containing microparticles in atherothrombosis. *Circ Res* 2000;**87:**81–2.
[9] Schecter AD, Giesen PL, Taby O, Rosenfield CL, Rossikhina M, Fyfe BS, et al. Tissue factor expression in human arterial smooth muscle cells. TF is present in three cellular pools

after growth factor stimulation. *J Clin Invest* 1997;**100:** 2276–85.
[10] Colucci M, Balconi G, Lorenzet R, Pietra A, Locati D, Donati MB, et al. Cultured human endothelial cells generate tissue factor in response to endotoxin. *J Clin Invest* 1983;**71:** 1893–6.
[11] Drake TA, Ruf W, Morrissey JH, Edgington TS. Functional tissue factor is entirely cell surface expressed on lipopolysaccharide-stimulated human blood monocytes and a constitutively tissue factor-producing neoplastic cell line. *J Cell Biol* 1989;**109:**389–95.
[12] Siddiqui FA, Desai H, Amirkhosravi A, Amaya M, Francis JL. The presence and release of tissue factor from human platelets. *Platelets* 2002;**13:**247–53.
[13] Maynard JR, Dreyer BE, Stemerman MB, Pitlick FA. Tissue-factor coagulant activity of cultured human endothelial and smooth muscle cells and fibroblasts. *Blood* 1977;**50:**387–96.
[14] Nieuwland R, Berckmans RJ, Rotteveel-Eijkman RC, Maquelin KN, Roozendaal KJ, Jansen PGM, et al. Cell-derived microparticles generated in patients during cardiopulmonary bypass are highly procoagulant. *Circulation* 1997;**96:** 3534–41.
[15] Berckmans RJ, Nieuwland R, Tak PP, Böing AN, Romijn FP, Kraan MC, et al. Cell-derived microparticles in synovial fluid from inflamed arthritic joints support coagulation exclusively via a factor VII-dependent mechanism. *Arthritis Rheum* 2002;**46:**2857–66.
[16] Nieuwland R, Berckmans RJ, McGregor S, Böing AN, Romijn FPHTM, Westendorp RGJ, et al. Cellular origin and procoagulant properties of microparticles in meningococcal sepsis. *Blood* 2000;**95:**930–5.
[17] Biró E, Sturk-Maquelin KN, Vogel GM, Meuleman DG, Smit MJ, Hack CE, et al. Human cell-derived microparticles promote thrombus formation in vivo in a tissue factor-dependent manner. *J Thromb Haemost* 2003;**1:**2561–8.
[18] Kagawa H, Komiyama Y, Nakamura S, Miyaka T, Miyazaki Y. Expression of functional tissue factor on small vesicles of lipopolysaccharide-stimulated human vascular endothelial cells. *Thromb Res* 1998;**91:**297–304.
[19] Combes V, Simon AC, Grau GE, Arnoux D, Camoin L, Sabatier F, et al. In vitro generation of endothelial microparticles and possible prothrombotic activity in patients with lupus anticoagulant. *J Clin Invest* 1999;**104:**93–102.
[20] Abid Hussein MN, Meesters EW, Osmanovic, Romijn FP, Nieuwland R, Sturk A. Antigenic characterization of endothelial cell-derived microparticles and their detection ex vivo. *J Thromb Haemost* 2003;**1:**2434–43.
[21] Jimenez JJ, Jy W, Mauro LM, Horstman LL, Ahn YS. Elevated endothelial microparticles in thrombotic thrombocytopenic purpura: findings from brain and renal microvascular cell culture and patients with active disease. *Br J Haematol* 2001;**112:**81–90.
[22] Brogan PA, Shah V, Brachet C, Harnden A, Mant D, Klein N, et al. Endothelial and platelet microparticles in vasculitis of the young. *Arthritis Rheum* 2004;**50:**927–36.
[23] Simak J, Holada K, Risitano AM, Zivny JH, Young NS, Vostal JG. Elevated circulating endothelial membrane microparticles in paroxysmal nocturnal haemoglobinuria. *Br J Haematol* 2004;**125:**804–13.
[24] Minagar A, Jy W, Jimenez JJ, Sheremata WA, Mauro LM, Mao WW, et al. Elevated plasma endothelial microparticles in multiple sclerosis. *Neurology* 2001;**56:**1319–24.
[25] Ferreira AC, Peter AA, Mendez AJ, Jimenez JJ, Mauro LM, Chirinos JA, et al. Postprandial hypertriglyceridemia increases circulating levels of endothelial cell microparticles. *Circulation* 2004;**110:**3599–603.
[26] Smeets EF, Comfurius P, Bevers EM, Zwaal RF. Contribution of different phospholipid classes to the prothrombin converting capacity of sonicated lipid vesicles. *Thromb Res* 1996; **81:**419–26.
[27] Neuenschwander PF, Bianco-Fisher E, Rezaie AR, Morrissey JH. Phosphatidylethanolamine augments factor VIIa-tissue factor activity: enhancement of sensitivity to phosphatidylserine. *Biochemistry* 1995;**34:**13988–93.
[28] Smith SA, Morrissey JH. Properties of recombinant human thromboplastin that determine the International Sensitivity Index (ISI). *J Thromb Haemost* 2004;**2:**1610–6.
[29] Biró E, Akkerman JW, Hoek FJ, Gorter G, Pronk LM, Sturk A, et al. The phospholipid composition and cholesterol content of platelet-derived microparticles: a comparison with platelet membrane fractions. *J Thromb Haemost* 2005; **3:**2754–63.
[30] Berckmans RJ, Nieuwland R, Böing AN, Romijn FP, Hack CE, Sturk A. Cell-derived microparticles circulate in healthy humans and support low grade thrombin generation. *Thromb Haemost* 2001;**85:**639–46.
[31] Vogel GM, Meuleman DG, Bourgondien FG, Hobbelen PM. Comparison of two experimental thrombosis models in rats effects of four glycosaminoglycans. *Thromb Res* 1989;**54:** 399–410.
[32] Abid Hussein MN, Nieuwland R, Hau CM, Evers LM, Meesters EW, Sturk A. Cell-derived microparticles contain caspase 3 in vitro and in vivo. *J Thromb Haemost* 2005;**3:** 888–96.
[33] Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. *Can J Biochem Physiol* 1959;**37:** 911–7.
[34] Weerheim AM, Kolb AM, Sturk A, Nieuwland R. Phospholipid composition of cell-derived microparticles determined by one-dimensional high-performance thin-layer chromatography. *Anal Biochem* 2002;**302:**191–8.
[35] Jimenez JJ, Wenche J, Lucia MM, Soderland C, Horstman LL, Ahn YS. Endothelial cells release phenotypically and quantitatively distinct microparticles in activation and apoptosis. *Thromb Res* 2003;**109:**175–80.
[36] Del Conde I, Shrimpton CN, Thiagarajan P, Lopez JA. Tissue-factor-bearing microvesicles arise from lipid rafts and fuse with activated platelets to initiate coagulation. *Blood* 2005;**106:**1604–11.
[37] Drake TA, Cheng J, Chang A, Taylor Jr FB. Expression of tissue factor, thrombomodulin, and E-selectin in baboons with lethal *Escherichia coli* sepsis. *Am J Pathol* 1993;**142:** 1458–70.