Leukocyte- and endothelial-derived microparticles: a circulating source for fibrinolysis

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ABSTRACT

Background
We recently assigned a new fibrinolytic function to cell-derived microparticles *in vitro*. In this study we explored the relevance of this novel property of microparticles to the *in vivo* situation.

Design and Methods
Circulating microparticles were isolated from the plasma of patients with thrombotic thrombocytopenic purpura or cardiovascular disease and from healthy subjects. Microparticles were also obtained from purified human blood cell subpopulations. The plasminogen activators on microparticles were identified by flow cytometry and enzyme-linked immunosorbent assays; their capacity to generate plasmin was quantified with a chromogenic assay and their fibrinolytic activity was determined by zymography.

Results
Circulating microparticles isolated from patients generate a range of plasmin activity at their surface. This property was related to a variable content of urokinase-type plasminogen activator and/or tissue plasminogen activator. Using distinct microparticle subpopulations, we demonstrated that plasmin is generated on endothelial and leukocyte microparticles, but not on microparticles of platelet or erythrocyte origin. Leukocyte-derived microparticles bear urokinase-type plasminogen activator and its receptor whereas endothelial microparticles carry tissue plasminogen activator and tissue plasminogen activator/inhibitor complexes.

Conclusions
Endothelial and leukocyte microparticles, bearing respectively tissue plasminogen activator or urokinase-type plasminogen activator, support a part of the fibrinolytic activity in the circulation which is modulated in pathological settings. Awareness of this blood-borne fibrinolytic activity conveyed by microparticles provides a more comprehensive view of the role of microparticles in the hemostatic equilibrium.

Key words: fibrinolytic microparticles, plasmin, plasminogen, uPA; tPA.

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Introduction

Microparticles are small vesicles resulting from membrane blebbing of most activated or apoptotic cells. Microparticles derived from blood and vascular cells can be detected in the circulation and over the past decade have become accepted as diagnostic and prognostic biomarkers in cardiovascular and inflammatory diseases, and in cancer.[1] Accumulating evidence indicates that beyond their clinical relevance as biomarkers, microparticles also convey various bioactive effectors from the parental cell. Previous *in vitro* and *in vivo* studies showed that microparticles propagate a spectrum of biological activities and are involved in many different processes, such as activation of coagulation, inflammation, vascular remodeling and angiogenesis.[2-6] They bear tissue factor-dependent procoagulant activity and regulate procoagulant pathways in monocytes.[7] They also carry cytokines important for inflammation[8,9] and participate in endothelial dysfunction by decreasing the production of nitric oxide.[4] Furthermore, it has been shown that endothelial microparticles have matrix metalloproteinase activity on their surface, suggesting that they could participate in extracellular matrix degradation, vascular remodeling and angiogenesis.[3]

We previously assigned a hitherto unreported fibrinolytic function to microparticles[10] and, more recently, we demonstrated that microparticles participate in a newly identified mechanism of fibrinolytic cross-talk.[11] In these *in vitro* studies, performed with microparticles derived from the human microvascular endothelial cell line, HMEC-1, we demonstrated that these endothelial microparticles constitute a catalytic surface for efficient activation of plasminogen by the urokinase-type plasminogen activator (uPA) anchored to its receptor, uPAR.[10] Interestingly, these microparticles may also activate plasminogen bound to fibrin, extracellular matrix proteins or platelets.[11] The possibility that circulating microparticles also serve as a template for plasmin formation and fibrinolytic activity *in vivo* and their cellular origin remain unsolved issues that are the object of the present study.

Design and Methods

Isolation of microparticles from human plasma

Platelet-poor plasma was separated (1500 *g*, 15 min) from blood collected into 0.119 M sodium citrate from healthy volunteers (n=36) and from patients with systemic lupus erythematosus (n=22), diagnosed according to the American College of Rheumatology criteria,[12] or with atherosclerotic complications (n=16) involved in a previous study.[13] Citrated plasma was also obtained by plasmapheresis from patients suffering from acute thrombotic thrombocytopenic purpura (TTP, n=10). Informed consent was obtained from all patients according to the Declaration of Helsinki.

Microparticles were isolated by sequential centrifugation of platelet-poor plasma, first at 12000 *g* for 2 min to remove residual platelets. The platelet-free plasma was then centrifuged at 20000 *g* for 90 min at 4°C. Centrifugation at 100000 *g*, used in some studies, was avoided to prevent concomitant sedimentation of exosomes, a different type of membrane vesicles.[14,15] Pelleted microparticles were washed twice (20000 *g* for 90 min at 4°C) and re-suspended in phosphate-buffered saline. In some experiments, circulating microparticles were depleted of erythrocyte and platelet microparticles by magnetic immuno-separation using beads coated with CD41 and CD235a antibodies. Flow cytometry was used to confirm that these two types of microparticles had been removed (with a reduction of least 90%) (*Online Supplementary Data, Section I, Figure S1*). Control experiments were performed in parallel using beads coated with irrelevant antibodies.

Generation of microparticles from blood cells

To investigate which subtype of circulating microparticles may support plasminogen activation activity, microparticles were generated *in vitro* from purified blood cell types. For this purpose, whole blood from healthy volunteers or patients, who had not taken anti-platelet medication for at least 2 weeks, was collected into 0.119 sodium citrate tubes.

Human platelets, prepared as described elsewere,[16] were incubated with 1 NIH U/mL of thrombin and/or 1 μM of ionophore A23187 (Sigma, St. Louis, MO, USA), and/or 10 μg/mL of collagen (Stago, Asnière, France), for 15 min at 37°C without stirring. Platelets were subsequently pelleted by centrifugation, first at 1500 *g* for 15 min and then, after the residual platelets had been discarded, by centrifugation at 12000 *g* for 2 min. The microparticles were then isolated from the supernatant and washed as described above. Flow cytometry, was used to check that the purity was superior to 95%.

Erythrocyte microparticles were generated as previously described by Salzer *et al.* with minor modifications.[17] Red blood cells pelleted at 200 *g* for 10 min and subsequently washed in sodium chloride 0.9%, were suspended in nine volumes of phosphate-buffered saline containing 1 mM $CaCl_2$ and 5 μM of A23187 and incubated at 37°C for 30 min. Erythrocytes were subsequently separated by centrifugation at 12000 *g* for 2 min, and the microparticles were isolated and washed as described above. Flow cytometry was used to check that the purity was superior to 95%.

Leukocyte microparticles were prepared as previously described with minor modifications.[18] Peripheral blood mononuclear cells were isolated from buffy coats by Ficoll™ (Lymphocyte separation medium 1077). Monocytes were isolated by $CD14^+$ immuno-magnetic separation on magnetic sphere columns (Miltenyi Biotec, Bergish Gladbach, Germany). Flow cytometry was used to check that the purity was superior to 95%. Monocytes were stimulated by lipopolysaccharides (1 μg/mL) overnight. Vesiculation was evaluated by flow cytometry using staining with annexin A5-fluorescein isothyiocyanate (FITC)/CD11b. Neutrophils were purified from buffy coats and were stimulated by N-formylmethionyl-leucyl-phenylalanine (1 μM) for 2 h at 37°C. Lymphocytes were purified from blood mononuclear cells and were stimulated by platelet-activating factor 500 nM/ phorbol 12-myristate 13-acetate 50 nM for 2 h at 37°C. Microparticles were isolated and washed as described above.

Generation and harvesting of microparticles from endothelial cells

Several subtypes of human endothelial cells originating from distinct vascular beds were purchased from Clonetics (Grand Island, NY, USA) and cultured in 0.2% gelatin-coated flasks in EGM2-MV medium: renal artery endothelial cells were used at passage 8, coronary artery endothelial cells at passage 7 and both dermal human microvascular vein endothelial cells and human pulmonary artery endothelial cells at passage 6. Endothelial microparticles were purified from culture medium conditioned by sub-confluent endothelial cells stimulated or not with 10 ng/mL tumor necrosis factor-α (PeproTech Inc, Rocky Hill, NJ, USA) for 24 h, as previously described with some modifications.[19] Culture supernatants were centrifuged at 300 *g* for 5 min and at 2500 *g* for 10 min to remove detached cells and debris. The microparticles

were isolated as described above, washed twice and re-suspended in HEPES buffer.

Characterization of microparticles by flow cytometry and enzyme-linked immunosorbent assays

Aliquots (10 μL) of microparticle suspensions, diluted 1/10, were labeled using FITC-conjugated annexin A5 (Abcys, Paris, France) or specific monoclonal antibodies. Phycoerythrin (PE)-labeled anti-CD41, FITC anti-CD31, PE anti-CD11b and PE anti-CD235a were from Beckman Coulter (Marseille, France). Flow cytometry analysis (*Online Supplementary Data Section II, Figure S2*) was performed on a Cytomics FC500® instrument (Beckman Coulter, Miami, FL, USA). Microparticles were analyzed as previously described.[20,21]

The presence of uPA, uPAR and tissue plasminogen activator (tPA) was evaluated by flow cytometry using FITC-labeled antibodies on microparticles gated according to positivity for CD146-PE (endothelial microparticles), CD59-PE (endothelial, erythrocyte and leukocyte microparticles) or CD41-PE (platelet microparticles) (*Online Supplementary Figure S3*). Antibodies matched for protein concentration and fluorescence/protein ratio were used as controls.

Pelleted microparticles were lysed in 100 mM Tris-HCl buffer pH 8.1, containing 0.5% Triton X-100 and supplemented with a mixture of complete protease inhibitors (Roche Diagnostic GmbH, Mannheim, Germany). Concentrations of uPA and uPAR were assayed by enzyme-linked immunosorbent assay (ELISA) (894 and 893 IMUBIND® ELISA kits, American Diagnostica, Greenwich, CT, USA) according to the manufacturer's instruction. The results are expressed as nanograms of protein per 10^6 microparticles. The tPA and plasminogen activator inhibitor type-1 (PAI 1) content were measured by ELISA (Asserachrom® tPA and Asserachrom® PAI-1, Diagnostica Stago, Asnière, France). These results are also expressed as nanograms of protein per 10^6 microparticles.

Determination of plasminogen activators, plasminogen activation and fibrinolytic activity on microparticles

The presence and identity of plasminogen activators and their inhibitors borne by microparticles were analyzed by direct and reverse fibrin autography following sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) performed as described previously.[22] Briefly, microparticles were lysed in 100 mM Tris-HCl buffer, pH 8.1, containing 1% Triton X-100. Microparticle lysates (10 μL from $2x10^5$ microparticles) and reference proteins (10 μL of tPA 5 nM, uPA 1 nM and plasmin 500 nM) were electrophoresed in a 7.5% polyacrylamide gel under non-reducing conditions. SDS was then exchanged with 2.5% Triton X-100. After washing off excess Triton X-100 with distilled water, the gel was carefully overlaid on a 1% agarose gel containing 1 mg/mL bovine fibrinogen, 100 nM plasminogen and 0.2 NIH U/mL of bovine thrombin. For reverse fibrin zymography, the fibrin gel was supplemented with 0.05 IU/mL of urokinase. Zymograms were allowed to develop at 37°C for 24 h and photographed at regular intervals using dark-ground illumination. Active proteins in microparticle lysates were identified by reference to the migration of known markers (uPA, tPA, plasmin). When required the fibrin-agarose indicator gel was supplemented with antibodies (10 μg/mL) directed against specific plasminogen activators.

The capacity of microparticles to activate plasminogen was determined by incubating a fixed concentration of plasminogen (1 μM) with the microparticles in the presence of a chromogenic substrate selective for plasmin (CBS 0065, 0.75 mM final concentration), as described previously.[10] Plasmin formed from plasminogen bound at the surface of microparticles cleaves the chromogenic substrate and the released p-nitroaniline is detected by measuring A_{405nm} as a function of time. Results are expressed in $A_{405nm}x10^{-3}$/min. When indicated, the following inhibitors were pre-incubated with the microparticles: 10 μg/mL goat anti-human tPA (Biopool, Uppsala, Sweden), 20 μg/mL mouse anti-human uPA (American Diagnostica 3940), and respective irrelevant control IgGs (Biocytex, Marseille, France); amiloride and ε-aminocaproic acid (Sigma-Aldrich) were used at the final concentrations of 100 μM and 0.05 M, respectively.

To scale the degree of plasmin formation by microparticles, standard curves, constructed with reference microparticles (200000 per 50 μL) bearing known molar amounts of uPA or tPA (THP1- or CHO-derived microparticles, respectively), were used (*Online Supplementary Data Section III, Figures S4 and S5*).

The fibrinolytic activity of microparticle-free plasma from patients and controls was estimated by measuring the amount of plasmin formed on a fibrin surface in contact with the plasma euglobulin fraction in the presence of CBS 0065 (0.75 mM final concentration).[23]

Statistical analysis

Statistical analysis was performed with KaleidaGraph software (Synergy Software, Reading, PA, USA). Significant differences were determined using the non-parametric Mann-Whitney test. A *P* value less than 0.05 was considered statistically significant.

Results

In vivo evidence of blood-borne fibrinolytic activity supported by microparticles: identification of plasminogen activators

To investigate the possibility that blood-borne microparticles may be involved in plasmin generation under conditions occurring *in vivo*, we studied microparticles from healthy controls and patients with pathological states reported to be associated with increased microparticle concentration, namely atherosclerotic vascular disease (n=16) and systemic lupus erythematosus (n=22). Microparticles were isolated by centrifuging platelet-free plasma at 20000 *g* for 90 min. Thus, none of the experiments presented in this paper included exosomes and all concern exclusively microparticles, as indicated by nanoparticle tracking analysis (*Online Supplementary Data Section IV, Figure S6*). As illustrated in Figure 1, it was possible to detect varying levels of plasminogen activator activity on microparticles in both controls and patients. Interestingly, the plasminogen activator activity of microparticles was significantly higher in the two groups of patients compared to healthy controls [median (25^{th}-75^{th} percentiles): 1.2 (0.6-1.93) mOD/min and 0.87 (0.61-1.59) mOD/min *versus* 0.5 (0.4-0.6) mOD/min, $P<0.003$ and $P<0.007$, respectively). However, about 50% of patients had a plasminogen activator activity similar to that in healthy controls.

The nature of plasminogen activators involved in the fibrinolytic activity was analyzed using samples obtained by plasmapheresis from patients with acute TTP, a pathological situation in which there is increased vesiculation.[24,25] Plasmapheresis has the advantage over classical blood sampling of providing sufficient amounts of plasma for microparticle isolation and extensive characterization. All isolated microparticle samples (n=10) generated plasmin activity upon incubation with 1 μM plasminogen. Figure

2A shows plasmin generation (0.7-5.9 mOD/min) in TTP samples ($2x10^5$ microparticles/well) which display variable plasminogen activator activity (control in the absence of microparticles: 0.015 mOD/min). The inhibition of plasminogen binding and plasmin generation by ε-aminocaproic acid was consistent with a lysine-dependent mechanism for plasminogen binding and activation at the surface of the microparticles (Figure 2B). The plasminogen activator activity in distinct samples (Figure 2C upper panel) was identified as uPA (TTP 6) or tPA (TTP 7) in fibrin-agarose gels, as indicated by the position of their fibrinolytic bands relative to known markers. For instance, the single fibrinolytic band visible in sample TTP 6 and the corresponding uPA standard were absent in a fibrin-agarose gel containing anti-uPA antibodies (Figure 2C, lower panel). This antibody also inhibited plasmin formation by microparticles from TTP 4 in a chromogenic assay (Figure 2B). The microparticle sample TTP 7 in Figure 2C produced fibrinolytic bands of increased intensity corresponding to the position of uPA, tPA and tPA in complex with its inhibitor. The uPA fibrinolytic band did not appear in the presence of anti-uPA antibodies whereas tPA and its complexes retained their activity (Figure 2C, lower panel). In two additional TTP samples it was possible to quantify the molar activity of plasminogen activators on $2x10^5$ microparticles/50 µL (tPA: 1.93 and 0.84 pM; uPA 1.5 and 0.83 pM). For this purpose we used reference microparticles bearing known molar concentrations of either tPA or uPA and amiloride to resolve tPA activity (*Online Supplementary Data Section III*). Free tPA and tPA in complex with its inhibitor were also identified in samples from patients with systemic lupus erythrematosus (*Online Supplementary Data Section V, Figure S7*). These results provide the first evidence that human circulating microparticles carry plasminogen activators and activate membrane-bound plasminogen. These *ex vivo* data suggest that blood-borne microparticles may generate and disseminate plasmin activity *in vivo*.

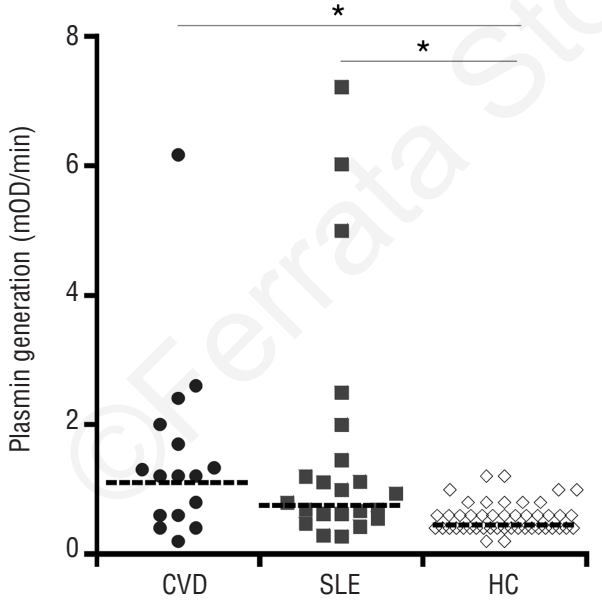

Figure 1. Plasmin generated by circulating microparticles from patients with cardiovascular disease (CVD), systemic lupus erythematosus (SLE) and healthy controls (HC). Microparticles ($2x10^5$/well) were isolated as indicated in the *Design and Methods section* and then incubated with plasminogen (1 µM) in the presence of a plasmin-selective chromogenic substrate (CBS 0065, 0.75 mM). Dots represent the amount of plasmin formed (mOD/min). Results represent the median (25^{th}-75^{th} percentile) of a duplicated experiment. The median for each category is represented by the dotted lines. ● CVD 1.2 (0.6-1.93) mOD/min and ■ SLE 0.87 (0.61-1.59) mOD/min *versus* ❋ HC 0.5 (0.4-0.6) mOD/min, *P<0.01.

The fibrinolytic activity in circulating blood is borne by leukocyte- and endothelium-derived microparticles

To explore the fibrinolytic activity of microparticles further, we determined the cellular origin of the microparticles with plasminogen activator activity. We first performed experiments using specific antibodies coated on magnetic beads to deplete platelet and erythrocyte microparticles, the major subpopulations of circulating microparticles, from the samples. After this procedure, the residual platelet and erythrocyte microparticles constituted only about 10% of the microparticles in the depleted samples (Figures 3A, 3B). The predominant subpopulations of endothelial and leukocyte microparticles in the depleted sample generated significantly more plasmin formation compared to the non-depleted sample (Figure 3C). These data suggest that: (i) endothelial and leukocyte microparticles provide the real support to plasminogen activator activity, and (ii) platelet and erythrocyte microparticles do not contribute to the fibrinolytic activity of microparticles isolated from circulating blood. Of note, platelets and platelet microparticles bear active PAI-1 (Figure 3D). Thus, the unexpected increase in plasmin formation observed in the depleted samples was probably related to the absence of platelet microparticles bearing active PAI-1.

To identify which plasminogen activators are borne specifically by microparticles of endothelial and leukocyte origin, we studied the fibrinolytic activity of isolated microparticles ($2x10^5$/well) derived from distinct purified blood cell subpopulations and from primary human endothelial cells.

Endothelial-derived microparticles

Plasmin generation was detected on endothelial microparticles with variations according to their anatomical origin (Figure 4A). The greatest and least fibrinolytic activities were displayed by endothelial microparticles of renal artery (1.2±0.2 mOD/min) and dermal microvascular origin (0.03±0.01 mOD/min), respectively. By zymography, two fibrinolytic bands resistant to anti-uPA antibodies were observed in endothelial microparticle lysates (Figure 4B). The lower band corresponded to the migration of purified tPA whereas the top band, of higher molecular weight, corresponded to the migration of tPA in complex with its inhibitor (Cs in Figure 4B), in agreement with previous findings.[22] Consistent with this observation, significant levels of PAI-1 antigen were detected in lysates of endothelial microparticles with a specific ELISA (Figure 4C). The presence of tPA was further confirmed by ELISA (Figure 4D) and by flow cytometry (*Online Supplementary Figure S3*), whereas uPA activity and antigen were undetectable. The molarity of active tPA borne by endothelial microparticles was determined by reference to standard microparticles bearing known molar amounts of tPA (Table 1).

Leukocyte-derived microparticles

Plasminogen activator activity was also detected on leukocyte microparticles of monocyte, lymphocyte and

neutrophil origin (Figure 4A). The presence of such activity on leukocyte microparticles was further confirmed by a lytic band on zymography corresponding to the position of uPA (Figure 4B), which was absent in the presence of antibodies to uPA (*data not shown*). Flow cytometry (*Online Supplementary Figure S3*) and ELISA (Figure 4D) allowed detection of uPA and its receptor uPAR on the surface of leukocyte microparticles. In a parallel experiment, tPA antigen was undetectable on leukocyte microparticles (*Online Supplementary Figure S3*). The molarity of uPA borne by leukocyte microparticles was calculated using reference standard microparticles bearing known molar amounts of uPA (Table 2).

Platelet- and erythrocyte-derived microparticles.

Both platelet microparticles and erythrocyte microparticles (up to $5x10^6$ microparticles/well) failed to generated plasmin (Figure 4A) or fibrinolytic bands (Figure 4B). Accordingly, depletion of erythrocyte- and platelet-derived microparticles did not decrease the fibrinolytic activity of microparticles isolated from plasma (Figure 3C). Similar negative results regarding plasminogen activator activity were obtained when platelet microparticles were generated using different platelet agonist combinations (collagen, thrombin and A23187; *data not shown*). Flow cytometry experiments also failed to detect uPA or tPA antigens on platelet- or erythrocyte- derived microparticles (*Online Supplementary Figure S3*).

Collectively, these results indicate that active plasminogen activators expressed by endothelial cells and leukocytes (tPA and uPA, respectively) are conveyed in circulating blood by endothelial and leukocyte microparticles.

Discussion

Using the human microvascular endothelial cell line HMEC-1,[26] we previously reported that endothelial microparticles have fibrinolytic activity *in vitro* and participate in a fibrinolytic crosstalk.[10,11] However, the relevance of this model to fibrinolysis *in vivo* and whether microparticles derived from all circulating cell types support plasminogen activation remained unsolved questions that were addressed in the present study. First, we provided the proof of concept that microparticles produced *in vivo* support fibrinolysis as indicated by the formation of active plasmin on microparticles isolated from the blood of healthy subjects and patients with vascular diseases. Secondly, we showed that specific plasminogen activators

Table 1. tPA activity on endothelial cell-derived microparticles.

200 000 microparticles /50 μL	tPA (pM)
Renal artery endothelial cells	7.6±1.0
Coronary artery endothelial cells	3.9±0.1
Human microvascular endothelial cells	1.2±0.2

Table 2. uPA activity on leukocyte-derived microparticles.

200 000 microparticles /50 μL	uPA (pM)
Neutrophils	0.5±0.07
Lymphocytes	0.2±0.06
Monocytes	0.4±0.03

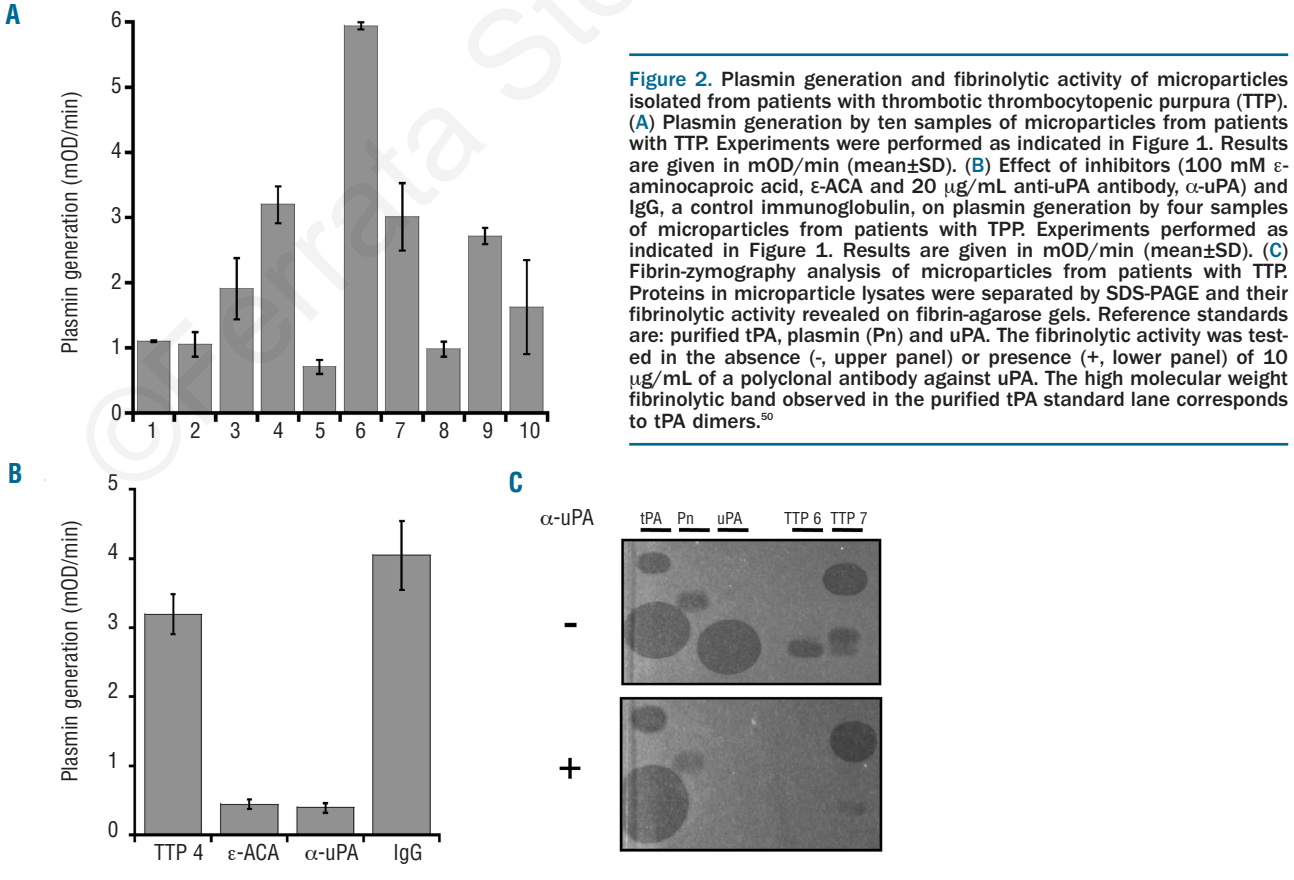

Figure 2. Plasmin generation and fibrinolytic activity of microparticles isolated from patients with thrombotic thrombocytopenic purpura (TTP). (A) Plasmin generation by ten samples of microparticles from patients with TTP. Experiments were performed as indicated in Figure 1. Results are given in mOD/min (mean±SD). (B) Effect of inhibitors (100 mM ε-aminocaproic acid, ε-ACA and 20 μg/mL anti-uPA antibody, α-uPA) and IgG, a control immunoglobulin, on plasmin generation by four samples of microparticles from patients with TPP. Experiments performed as indicated in Figure 1. Results are given in mOD/min (mean±SD). (C) Fibrin-zymography analysis of microparticles from patients with TTP. Proteins in microparticle lysates were separated by SDS-PAGE and their fibrinolytic activity revealed on fibrin-agarose gels. Reference standards are: purified tPA, plasmin (Pn) and uPA. The fibrinolytic activity was tested in the absence (-, upper panel) or presence (+, lower panel) of 10 μg/mL of a polyclonal antibody against uPA. The high molecular weight fibrinolytic band observed in the purified tPA standard lane corresponds to tPA dimers.[50]

on endothelial and leukocyte microparticles generate this fibrinolytic activity, whereas erythrocyte and platelet vesicles do not have this property. Thirdly, the plasminogen activators were identified as uPAR-bound uPA, on leukocyte microparticles, and tPA on endothelial microparticles. This study has, therefore, contributed to identifying new partners of blood-borne fibrinolytic activity.

We also provided evidence that microparticles with fibrinolytic activity are found at very low concentrations in healthy subjects whereas various levels of plasmin formation were found in different pathological situations (atherosclerosis, systemic lupus erythematosus and TTP). This varying level of *ex vivo* fibrinolytic activity reflects different amounts of active plasminogen activator borne by microparticles whereas the plasminogen activator activity of microparticle-free plasma was negligible, as reported previously.[23] The significance of these findings with regard to clinical status remains to be established in future studies. Nevertheless, we have revealed the existence of a microparticle-dependent profibrinolytic compensatory mechanism that may counterbalance the procoagulant phenotype and reduce the thrombotic risk in these patients.[24,27-29] These interesting features lay the basis for a potential new biomarker.

To identify microparticles that bear these plasminogen activators, we studied microparticle subpopulations released by primary endothelial cells in culture or by properly stimulated platelets, red cells and leukocytes isolated from circulating human blood. We provide here the first demonstration that plasminogen activators are borne in a selective manner by a minor fraction of microparticles circulating in human blood: endothelial microparticles carry tPA and leukocyte microparticles carry uPA/uPAR. This selectivity contrasts with the well-described procoagulant potential common to all microparticles.[30] Interestingly, we found various levels of fibrinolytic microparticles in differents pathological situations (atherosclerosis, systemic lupus erythematosus, TTP) that have been reported to be associated with increased levels of leukocyte and endothelial microparticles.[13,19,27,28,31-34] This fibrinolytic activity may reflect pathophysiological leuko-endothelial activation associated with inflammation, which could help to identify patients with a higher individual vascular risk in these clinical situations. Of note, leukocyte and endothelial

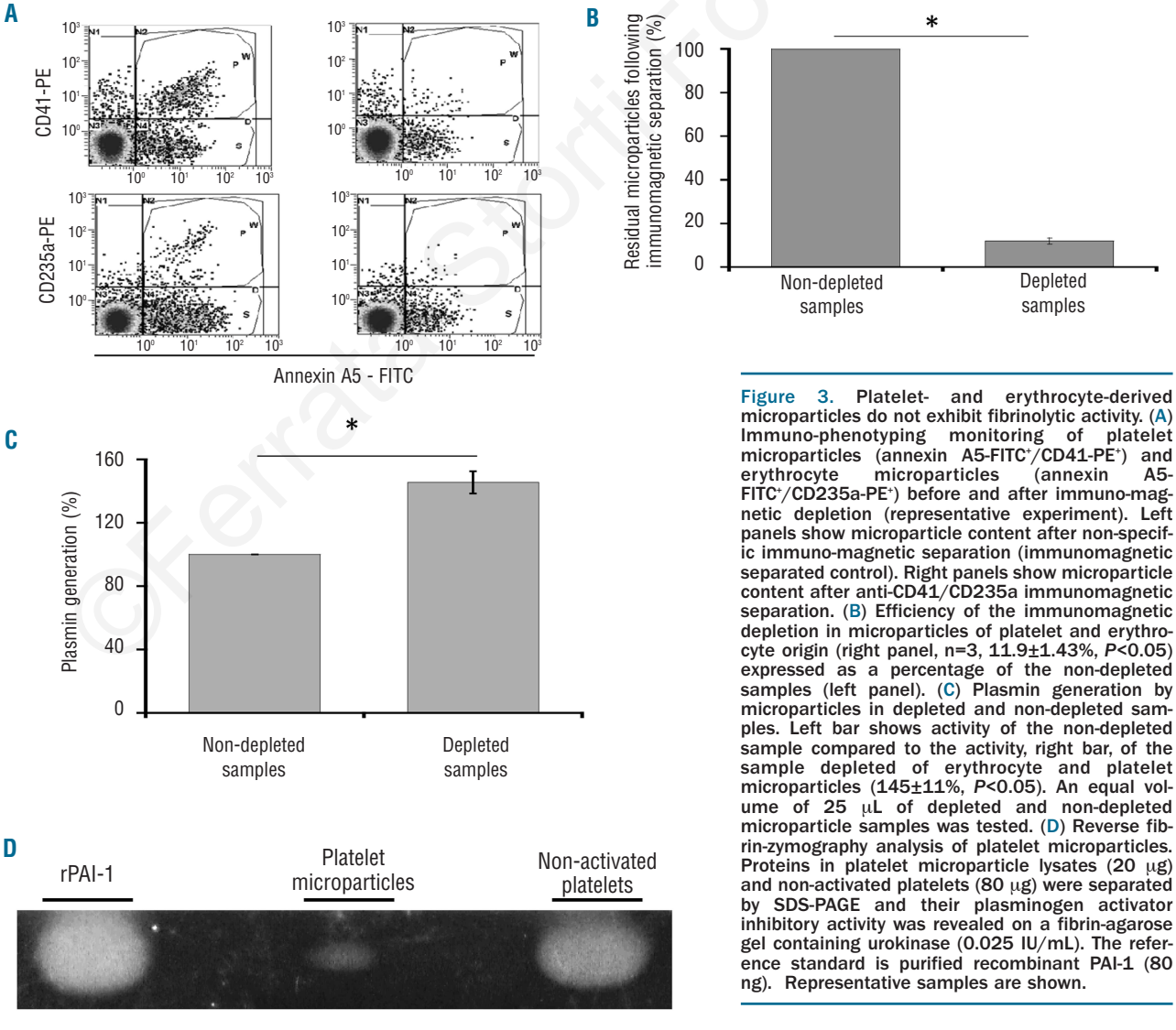

Figure 3. Platelet- and erythrocyte-derived microparticles do not exhibit fibrinolytic activity. (A) Immuno-phenotyping monitoring of platelet microparticles (annexin A5-FITC$^+$/CD41-PE$^+$) and erythrocyte microparticles (annexin A5-FITC$^+$/CD235a-PE$^+$) before and after immuno-magnetic depletion (representative experiment). Left panels show microparticle content after non-specific immuno-magnetic separation (immunomagnetic separated control). Right panels show microparticle content after anti-CD41/CD235a immunomagnetic separation. (B) Efficiency of the immunomagnetic depletion in microparticles of platelet and erythrocyte origin (right panel, n=3, 11.9±1.43%, P<0.05) expressed as a percentage of the non-depleted samples (left panel). (C) Plasmin generation by microparticles in depleted and non-depleted samples. Left bar shows activity of the non-depleted sample compared to the activity, right bar, of the sample depleted of erythrocyte and platelet microparticles (145±11%, P<0.05). An equal volume of 25 μL of depleted and non-depleted microparticle samples was tested. (D) Reverse fibrin-zymography analysis of platelet microparticles. Proteins in platelet microparticle lysates (20 μg) and non-activated platelets (80 μg) were separated by SDS-PAGE and their plasminogen activator inhibitory activity was revealed on a fibrin-agarose gel containing urokinase (0.025 IU/mL). The reference standard is purified recombinant PAI-1 (80 ng). Representative samples are shown.

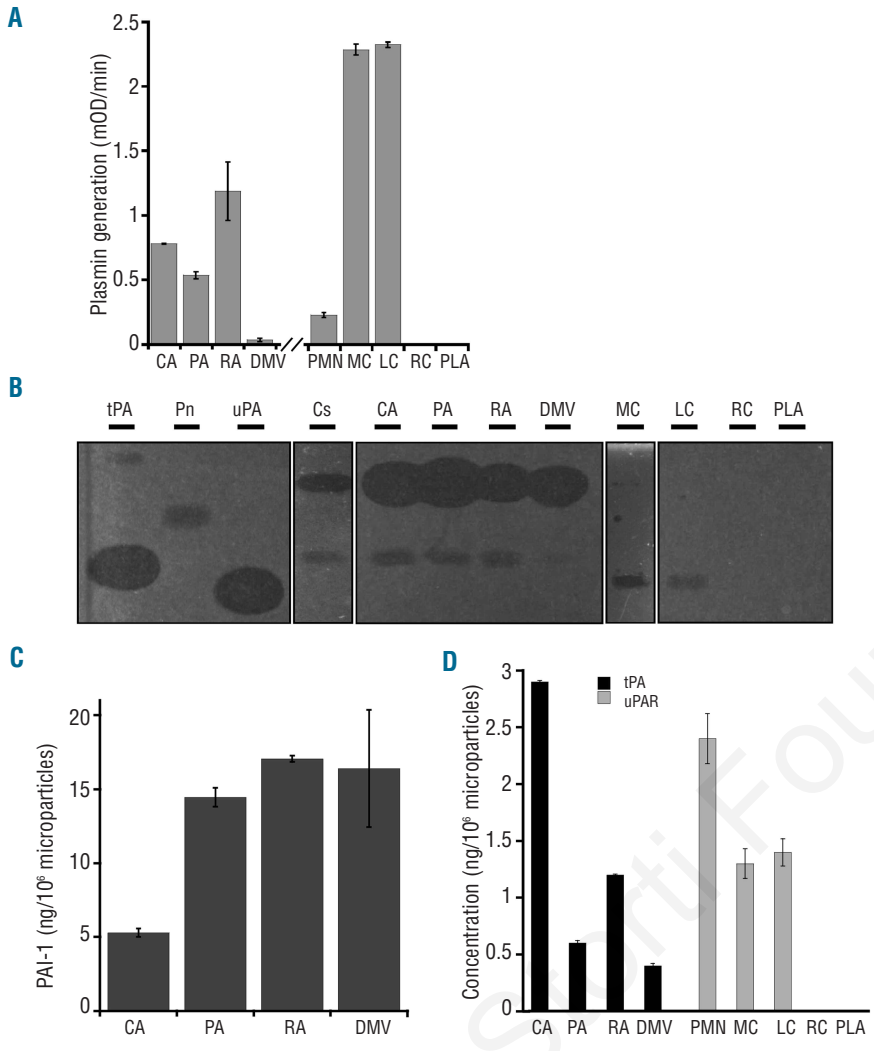

Figure 4. Plasminogen activators and fibrinolytic activity of human circulating blood cell- and primary endothelial cell-derived microparticles. Microparticles derived from circulating human platelets, leukocytes and erythrocytes, and from primary cultures of human endothelial cells were prepared and isolated as indicated in the *Design and Methods* section. Endothelial microparticles were obtained from primary cultures of coronary artery (CA), pulmonary artery (PA), renal artery (RA) and dermal microvascular (DMV) endothelial cells. Circulating blood cell-derived microparticles were obtained from isolated polymorphonuclear cells (PMN), monocytes (MC), lymphocytes (LC), red cells (RC) and platelets (PLA). (A) Plasmin generation by microparticles ($2x10^5$/well) was tested as indicated in Figure 1. Bars represent the amount of plasmin formed (mOD/min). Results are the mean±SD of duplicate experiments (n=3). (B) Fibrin-zymography analysis of endothelial and circulating microparticles. Zymography was performed as indicated in the *Design and Methods* section. Reference standards were purified tPA, plasmin (Pn), uPA and tPA in complex with PAI-1 (Cs). Representative samples are shown. (C) Concentration of PAI-1 as determined by ELISA in cell-derived microparticle lysates (10^6 endothelial microparticles per well). Results are the mean±SD of duplicate experiments. (D) Concentration of uPAR and tPA determined by ELISA in cell-derived microparticle lysates (10^6 endothelial microparticles per well). Results are the mean±SD of duplicate experiments.

microparticles are among the circulating subsets least accessible by current methodologies, such as flow cytometry, because of their low proportion, size distribution and limited specific markers. Thus, the measurement of this circulating fibrinolytic activity could provide additional, innovative information compared to that provided simply by the counts of these microparticle subsets.

The study of microparticle samples from patients with TTP demonstrated that plasmin may be generated by either uPA or tPA. By combining a set of tests (chromogenic, zymography, ELISA) and flow cytometry, we demonstrated that this activity was exclusive to microparticle subsets derived from leukocytes (expressing uPA) or endothelial cells (expressing tPA). These distinctive characteristics may be used advantageously to identify the origin of the plasmin generating capacity of circulating microparticles in patients. The presence of uPA on microparticles derived from the human microvascular endothelial cell line HMEC-1 described in our previous report[10] was due to the atypical synthesis of this plasminogen activator by the modified cell line, which was used as a model to generate microparticles after tumor necrosis factor-α stimulation.

In contrast to uPA-bearing microparticles derived from circulating leukocytes and tPA-bearing microparticles derived from human primary endothelial cells, we did not detect plasmin generation on erythrocyte or platelet microparticles. Accordingly, plasminogen activators have not been described on red blood cells and their expression by human platelets has not been clearly demonstrated, except for the ectopic production of uPA in the platelet Quebec syndrome.[35] It is, however, possible that platelet microparticles may, in some cases, develop plasminogen activator activity. Indeed, since platelets bind plasminogen,[36-38] platelet microparticles may be a source of substrate for enhanced fibrinolysis by single-chain uPA[39] via a fibrinolytic crosstalk mechanism we demonstrated recently.[11] However, active PAI-1/vitronectin complexes present in platelets[40] and active PAI-1 detected in platelet microparticles as shown in this study, may modulate this fibrinolytic activity. Given that both platelet and erythrocyte microparticle subpopulations account for the bulk of circulating microparticles,[13] it was relevant to demonstrate the effect of their depletion from the pool of microparticles isolated from plasma. The unexpected increased fibrinolytic activity generated by the depleted samples may reflect the absence of platelet microparticles bearing active PAI-1.

Thus, we found that microparticles of endothelial and

leukocyte origin are the main sources of the plasmin generating capacity on microparticles in human plasma. However, in pathological settings, it remains possible that microparticles from other origins may have proteolytic activity in plasma. For instance, tumor microparticles were proven to circulate and have been associated with thrombosis due to the tissue factor they convey.[41,42] Microparticles from tumor cell lines and from ascites of cancer origin were found to bear vesicle-associated proteolytic activity represented by matrix metalloproteinases 2 and 9 and plasminogen activators.[43-47] The discovery of fibrinolytic microparticles that circulate in human plasma raises the possibility of detecting such activity in some patients with cancer, with potential diagnostic relevance or prognostic value in metastatic evolution.

Beyond fibrinolysis, plasmin formation by microparticles bearing the uPA/uPAR system may participate, in concert with matrix metalloproteinases, in other proteolytic functions.[48] Thus, the proteolytic activity of uPA-bearing microparticles may be of relevance in cell migration, angiogenesis and outside-in signal transduction. Indeed, we have previously shown that tube formation was stimulated by low concentrations of microparticles,[10] an effect that was related to crosstalk activation of plasminogen present in the Matrigel.[49] This pro-angiogenic effect is consistent with plasmin-associated proteolytic activity that favors cell migration via extracellular matrix processing. The role of microparticle-bound plasmin in pathological settings involving inflammation, atherosclerosis and angiogenesis remains to be investigated. The high concentration of microparticles reported in atherosclerotic plaques suggests that plasmin generation on microparticles could participate in the modulation of the cell apoptosis/angiogenesis balance influencing plaque vulnerability.

In summary, our study indicates that: (i) circulating human microparticles display a range of plasmin generation in various pathological conditions, (ii) this activity is specifically generated by tPA on endothelial microparticles and by uPA on leukocyte microparticles, and (iii) these microparticles support blood fibrinolytic activity. We have provided the first evidence that surface plasminogen activation is a functional feature of plasma microparticles and differs significantly differ between healthy subjects and subjects with various diseases. Collectively, these results suggest that the presence of profibrinolytic microparticles during clot formation anticipates their potential for clot lysis.

The existence of profibrinolytic microparticles in the circulation raises the question about the physiological relevance of this activity. Our data suggest that pro-fibrinolytic microparticles may compensate the effect of procoagulant microparticles. Further studies will be necessary to correlate the procoagulant *versus* the fibrinolytic effects of microparticles with the clinical status of patients. Awareness of this microparticle blood-borne fibrinolytic activity not only provides a more comprehensive view of the role of these microparticles in the hemostatic equilibrium but also lays the basis for a potential new biomarker.

Authorship and Disclosures

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