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To cite this version:

Tiphaine Dejouvencel, Loïc Doeuvre, Romaric Lacroix, Laurent Plawinski, Francoise Dignat-George, et al.. Fibrinolytic cross-talk: a new mechanism for plasmin formation.. Blood, 2010, 115 (10), pp.2048-56. 10.1182/blood-2009-06-228817 . inserm-00439535

HAL Id: inserm-00439535

https://inserm.hal.science/inserm-00439535v1

Submitted on 7 Jun 2010

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Fibrinolytic cross-talk: a new mechanism for plasmin formation

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Abstract

Fibrinolysis and pericellular proteolysis depend on molecular co-assembly of plasminogen and its activator on cell, fibrin or matrix surfaces. We report here the existence of a fibrinolytic cross-talk mechanism bypassing the requirement for their molecular co-assembly on the same surface. First, we demonstrate that despite impaired binding of Glu-plasminogen to the cell membrane by ε -aminocaproic acid (ε-ACA) or by a lysine-binding site-specific mAb, plasmin is unexpectedly formed by cell-associated urokinase (uPA). Second, we show that Glu-plasminogen bound to carboxy-terminal lysine residues in platelets, fibrin or extracellular matrix components (fibronectin, laminin) is transformed into plasmin by uPA expressed on monocytes or endothelial cell-derived microparticles but not by tissue-type plasminogen activator (tPA) expressed on neurons. A two-fold increase in plasmin formation was observed over activation on the same surface. Altogether, these data indicate that cellular uPA but not tPA expressed by distinct cells is specifically involved in the recognition of conformational changes and activation of Glu-plasminogen bound to other biological surfaces via a lysine-dependent mechanism. This uPA-driven cross-talk mechanism generates plasmin in situ with a high efficiency, thus highlighting its potential physiologic relevance in fibrinolysis and matrix proteolysis induced by inflammatory cells or cell-derived microparticles.

MESH Keywords 6-Aminocaproic Acid ; pharmacology ; Animals ; Antifibrinolytic Agents ; pharmacology ; Cell Communication ; physiology ; Cells, Cultured ; Extracellular Matrix ; drug effects ; metabolism ; Fibrinolysin ; metabolism ; Fibrinolysis ; drug effects ; physiology ; Humans ; Mice ; Plasminogen ; metabolism ; Plasminogen Activators ; metabolism ; Protein Processing, Post-Translational ; Receptor Cross-Talk ; drug effects ; physiology ; Signal Transduction ; drug effects ; physiology ; Urokinase-Type Plasminogen Activator ; metabolism

Author Keywords plasminogen ; urokinase ; lysine-binding site ; monocytes ; endothelial microparticles ; platelets

INTRODUCTION

Fibrinolysis and pericellular proteolysis depend on plasminogen activation at fibrin and cell surfaces, respectively. Plasminogen is a 92 kDa protein composed of 791 amino acids (Glu^1 - Asn^{791} , Glu-plasminogen) arranged in a serine protease region, five triple-loop structures (about 80 amino acid residues constrained by 3 disulfide bridges) called kringle (K) domains and an amino-terminal sequence (Glu^1 - Lys^{77} /Val^{79}).1 Domains K1 and K4 contain lysine-binding sites (LBS)2 that allow binding of plasminogen to cells,3 fibrin4 and extracellular matrix components5 with a moderate affinity (overall dissociation constant, Kd = 0.5 to 1 μM). This interaction is inhibited by carboxypeptidase B (CpB), an exopeptidase that targets the activation surface by cleaving exposed carboxy-terminal lysines (C-ter Lys), and by ε-aminocaproic acid (ε-ACA), a lysine analogue that targets the LBS of plasminogen.

Domain K5 of plasminogen contains a LBS of weak affinity that interacts with the amino-terminal peptide of Glu-plasminogen and favours a predominant compact “closed” conformation6 –8 over a very short-lived extended “open” form.8 , 9 Proteolytic removal of the amino-terminal peptide by plasmin (cleavage at either Lys^{62} , Arg^{68} or Lys^{77}) yields a stable truncated open form (Lys-plasminogen), unable to adopt the compact conformation.10 –12 In a similar manner, saturation of the weak lysine-binding site in Glu-plasminogen with ε-ACA causes a transition from the compact closed form to the open extended conformation8 , 9 characteristic of Lys-plasminogen.12 –14 Because ε-ACA is an analogue of C-ter Lys, it has been accepted that Glu-plasminogen adopts an “open” Lys-like conformation upon binding to C-ter Lys on surface biological receptors.8 , 9 Plasminogen binding to these receptors is a prerequisite for its efficient transformation into plasmin by a plasminogen activator localized on the same cell surface (either the tissue-type, tPA, or the urokinase-type, uPA, plasminogen activator). Quiescent endothelial cells and neurons characteristically express tPA15 , 16 whereas uPA is expressed by migrating capillary endothelial cells17 and inflammatory cells.18 It has recently been shown that a similar mechanism of plasmin formation is also functional at the surface of microparticles (EMP) derived from a human microvascular endothelial cell line.19

Plasmin formed on these cellular membranes is implicated either directly or via activation of matrix metalloproteinases, in proteolytic processing of extracellular matrix proteins,20 cell migration, angiogenesis and cell detachment-induced apoptosis.21 , 22

Previous studies have shown that uPA activates Lys-plasminogen and ε-ACA-liganded Glu-plasminogen faster than native Glu-plasminogen.10 , 11 , 23 –25 Furthermore, single-chain uPA (scuPA) specifically activates plasminogen bound to C-ter Lys in fibrin, 26 suggesting a relation between its molecular conformation and plasmin generation. We therefore hypothesized the existence of a new mechanism of plasmin formation bypassing the requirement for molecular co-assembly on the same surface, via a proteolytic cross-talk. In this study, we show that conformational changes induced in Glu-plasminogen by either ε-ACA or its binding to fibrin, platelets or matrix proteins are readily recognized by the uPA/uPAR system of either EMP, monocytes or THP-1 cells, but not by tPA-bearing cells. This cellular uPA-driven cross-talk mechanism results in a high efficiency in plasmin formation, thus highlighting its potential physiological relevance in fibrinolysis and extracellular matrix degradation. This novel mechanism may be an intermediary pathway in pathophysiologically relevant inflammatory processes such as atherothrombosis, angiogenesis and cell migration.

MATERIALS AND METHODS

Reagents

Human Glu- and Lys-plasminogen were purified as described27 and were over 99% pure as assessed by SDS-PAGE and by amino-terminal sequence analysis. Recombinant inactive human plasminogen with Ser^{741} mutagenized to Ala (r-Pg-Ala^{741}) and recombinant scuPA with Ile^{159} mutagenized to Gly (r-scuPA-Gly^{159}) were obtained and characterized as described.28 , 29 uPA and tPA (both over 99% single-chain form), a sheep antibody directed against uPA and an IgG_1 mAb 34D3 were obtained as described.20 ,30 The mAb 34D3 reacts with plasminogen fragment K1+2+3, blocks the LBS function of K1 and shows no cross-reaction with plasminogen K4. 31 The chromogenic substrate selective for plasmin (methylmalonyl)-hydroxyprolylarginine-para -nitroaniline (CBS0065) was from Stago (Asnieres, France). The lysine analogue ε-ACA, carboxypeptidase B (CpB, porcine pancreas), $ZnCl_2$, amiloride (A7410) and poly-L-lysine (P-9155) were from Sigma (St-Louis, MO, USA). D-Valyl-L-phenylalanyl-L-lysine chloro-methyl ketone (VFK) was from Calbiochem (La Jolla, USA). Surfactant-free white aliphatic amine latex beads were from Interfacial Dynamics Corp (Portland, USA).

Preparation of fibrin surfaces and immobilisation of matrix proteins

Fibrin surfaces were generated on polyglutaraldehyde-activated aliphatic amine latex beads or microtiter plates as described previously.27 The matrix proteins fibronectin and laminin (10 μg/mL) were immobilized onto polyglutaraldehyde-activated microtiter plates using a similar procedure.

To generate carboxy-terminal lysine residues on fibrin for the binding of plasminogen, the surfaces were treated with 10 nM plasmin for 15 min at 37°C. Plasmin was discarded and the surfaces treated with a 0.1 M phosphate buffer, pH 7.4, containing 1 μM VFK and 0.1 M ε-ACA to inhibit and elute remaining bound plasmin. The surfaces were then stored at 4°C in 50 mM phosphate buffer, pH 7.4, containing 80 mM NaCl, 0.2% bovine serum albumin, 0.05% Tween-20, 0.01% Azide and 1 μM VFK.

Cell culture

The human monocytic cell line THP-1 (American Type Culture Collection, Rockville, MD, USA) was grown in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), 4 mM glutamine, 0.5 mM sodium pyruvate, 0.5% non-essential amino acids, and 1% antibiotics (penicillin, streptomycin). Cortical neurons were obtained from Swiss mice embryos at 16 days of gestation, seeded in 96-well plates coated with 25 μg/mL poly-L-lysine, cultured using neurobasal medium (Invitrogen, Paisley UK) supplemented with 2% B27 (Invitrogen, Paisley UK) and 0.5 mM L-Glutamine as described.32 Neurons used for experiments 5 days after seeding contained less than 2.5% astrocytes.33 The human microvascular endothelial cell line (HMEC-1)34 (obtained from Dr. Ades, Centers for Disease Control, Atlanta, GA, USA) was cultured in MCDB 131 medium (Invitrogen, Paisly UK) supplemented with 10% MP-free FCS, 10 ng/mL human recombinant epidermal growth factor (Upstate Cell Signalling Solutions, Lake Placid, NY, USA) and 1 μg/mL hydrocortisone (Sigma, St Quentin Fallavier, France). Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 .

Isolation of monocytes and platelets

Peripheral blood mononuclear cells were isolated from citrated blood of healthy donors by Lymphoprep (Axis-Shield PoC AS, Oslo, Norway) separation at 600g for 20 min, at room temperature. Monocytes were then purified by cell sorting using CD14-coupled magnetic beads (Miltenyl Biotec, Bergisch-Gladbach, Germany), following the supplier's instructions. The purified cell population contained >98 % monocytes, as assessed by flow cytometry, using CD14 monoclonal antibodies (Immunotech, Marseille, France). Purified monocytes were allowed to adhere 2 h onto 96-well plates (Techno Plastic Products AC, Trasadingen, Switzerland).

Platelets were isolated from blood collected into acid-citrate-dextrose from healthy volunteers. Platelet-rich plasma was obtained by centrifugation at 200g for 15 min at room temperature, followed by a second centrifugation at 1 200g for 12 min to pellet platelets. Platelets were then washed twice in 36 mM citric acid buffer, pH 6.5, containing 100 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$ and 5 mM glucose.

Generation, harvesting and flow cytometry of endothelial microparticles

EMP were purifed from culture medium conditioned by sub-confluent HMEC-1 stimulated for 48 h with 100 ng/mL TNF-α (PeproTech Inc, Rocky Inc, Rocky Hill, NJ) as previously described with minor modifications.35 Culture supernatants from flasks were collected and cleared from detached cells or large cell fragments by centrifugation at 4 300g for 5 min. The supernatants were then centrifuged at 20 000g for 90 min at 4°C. Pelleted EMP were washed twice and re-suspended in phosphate-buffered saline (PBS). Aliquots of 10 μL EMP suspension, 1/100 diluted, were labeled using fluorescein isothiocyanate (FITC)-conjugated annexin V (Abcys, Paris, France) and EMP were quantitated by flow cytometry as previously described.36

Characterization of plasminogen activators in cell and microparticle extracts

THP-1 cells, cortical neurons, HMEC-1 cells, pelleted EMP and platelets were lysed in 100 mM Tris-HCl buffer, pH 8.1, containing 1 % Triton X-100. Lysates were clarified by centrifugation and protein concentrations were determined using the BCA kit (Pierce, Rockford, IL). Fibrin autography-electrophoresis was performed as described previously.37 Briefly, 10 μg of proteins in cell lysates and 10 μL of reference proteins (tPA 1 i.u/mL, uPA 3 i.u/mL and plasmin 200 nM) were electrophoresed in a 8% 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 support containing 1 mg/mL of bovine fibrinogen, 100 nM of bovine plasminogen and 0.2 NIH unit/mL of bovine thrombin. Zymograms were allowed to develop at 37°C during 24 h and photographed at regular intervals using dark-ground illumination. Active proteins in cell lysates were identified by reference to the migration of known markers (uPA, tPA, plasmin) and inhibition of their activity with specific antibodies incorporated into the fibrin-agar gel. The presence of uPA and uPAR on EMP was further identified both by electron microscopy and by flow cytometry as described.19

Effects of lysine-binding site ligands on plasmin formation by cells

Glu- or Lys-plasminogen at various concentrations (0 to 3 μM) was incubated at 37°C with uPA-bearing THP-1 cells or tPA-bearing cortical neurons, in the presence of 0.75 mM of CBS0065, a plasmin selective chromogenic substrate. The effect of various inhibitors (mAb anti-K1-LBS; amiloride, an uPA inhibitor; ε-ACA; PMSF-treated CpB) on plasminogen binding and activation was determined using a fixed final plasminogen concentration. Kinetics of plasmin formation was followed by measuring the release of p -nitroaniline from the chromogenic substrate, detected as a change in absorbance (ΔA_{405nm} /min), using a multiwell plate reader (MX5000, Dynex) thermostated at 37 °C. Rates of plasmin formation were calculated from the slopes of A_{405nm} versus time. After the activation reaction, the cells were washed in PBS and residual bound plasmin was detected by adding 0.75 mM CBS0065.

Activation of platelet-bound plasminogen by monocytes or neurons

Isolated platelets were treated with 5 nM plasmin for 30 min at room temperature in order to generate plasminogen-binding sites (carboxyterminal lysine residues) at their surface as reported previously.38 Platelets were then washed, treated with 1 μM (final concentration) aprotinin (Trasylol, Bayer) to inhibit residual plasmin and were finally incubated with 2 μM plasminogen for 30 min at room temperature. Excess unbound plasminogen was removed by washing.

Isolated monocytes and neurons used as a source of distinct plasminogen activator, respectively uPA and tPA, were plated at 10^5 cells/well in 96-well plates and treated with 50 μg/mL of CpB for 30 min at 37°C. The cells were then washed and incubated with varying amounts of plasminogen-bearing platelets (0 to 15×10^6 per well) in cell medium supplemented with 0.75 mM of the plasmin substrate CBS0065. The kinetics of plasmin formation on platelets was followed during 12 h by measuring the release of p -nitroaniline from the chromogenic substrate as a change in A_{405nm} as a function of time. The cells were then carefully rinsed with PBS to discard platelets. A volume of 100 μL of culture medium containing 0.75 mM CBS0065 was then added to determine if plasmin was present at the surface of monocytes or neurons.

Activation on plasminogen bound to fibrin-coated beads by monocytes, neurons or endothelial microparticles

Fibrin-coated beads were incubated with 1 μM plasminogen for 30 min at 37°C. Excess unbound plasminogen was removed by washing. Isolated monocytes, neurons and endothelial microparticles were treated with 50 μg/mL of CpB for 30 min at 37°C. Cells were then washed and incubated with 2.5×10^5 beads per well in cell medium supplemented with 0.75 mM of the plasmin substrate CBS0065. The kinetics of plasmin formation on fibrin-coated beads was followed during 12 h at 37°C by measuring the release of p- nitroaniline.

In parallel experiments, fibrin-coated beads were incubated with 1 μM native or recombinant active-site inactivated Glu-plasminogen (r-Pg-Ala^{741}) for 30 min at 37°C. Fibrin-coated beads with bound plasminogen were then incubated with 10^6 EMP in a final volume of 200 μL. After overnight incubation at 22°C, the fibrin-coated beads were sedimented by centrifugation and resuspended in 10 mM Tris-HCL pH 6.8 containing 10% SDS to elute fibrin-bound plasminogen derivatives. The supernatant was electrophoresed under non-reducing conditions, proteins were transferred to PVDF membranes and revealed with a HRP-conjugated mAb (150 ng/mL) directed against plasminogen K1. Purified plasmin was used as reference.

Activation of plasminogen bound to fibrin surfaces and matrix proteins by monocytes or endothelial microparticles

Glu-plasminogen at 1 μM was incubated with the fibrin surfaces or with immobilized fibronectin or laminin. In parallel experiments, fibronectin (50 μg/mL) and plasminogen (1 μM) were simultaneously incubated and allowed to bind to the surface of fibrin. Unbound proteins were discarded by washing and plasminogen bound to fibrin surfaces or to immobilized fibronectin and laminin was incubated with varying concentrations of either THP-1 cells or EMP in the presence of CBS0065. The transformation of bound plasminogen into plasmin was detected by measuring the release of p -nitroaniline as indicated above. After the activation reaction, the supernatant was collected, the plates were washed with PBS and matrix-bound plasmin detected by adding 0.75 mM of CBS0065. To quantitate degradation of the fibrin surface, the plates were again washed and then incubated with a mAb (FDP-14) that specifically recognizes fibrin fragmentation, as described.30 Degradation of fibronectin in the supernatant was detected by Western blot as described.22

Statistical analysis

Data are expressed as mean ± SEM. The statistics were performed using nonparametric tests of Kruskall Wallis and of Mann Whitney (Statview 5 software). Statistical significance was set at $p < 0.05$.

RESULTS

Plasminogen activation on cells

In order to illustrate the accepted mechanism of plasmin formation via co-assembly of plasminogen and its activators at the same cell surface, plasminogen activation experiments were performed on cultured cells. THP-1 and endothelial cells were shown to express active uPA at their membrane identified by fibrin zymography of cell lysates (Fig. 1A) on the basis of its molecular mass (Mr 54 000) and its inhibition with polyclonal anti-uPA IgG added to the indicator fibrin gel (not shown). Similar results were obtained with human monocytes (not shown). A fibrinolytic band corresponding to tPA (Mr 70 000) was detected in lysates of mouse cortical neurons. In contrast, neither tPA nor uPA activity could be detected in the lysates of human platelets. The presence of uPA and uPAR on EMP was demonstrated by immunoelectron microscopy and flow cytometry as described previously.19 THP-1 and cortical neurons in culture were able to activate plasminogen at their surface in a specific and dose-dependent manner until saturation was reached (Fig. 1B , THP-1: Km = 492 nM; neurons: Km = 49±9 nM, not shown), in agreement with previous studies.33 , 39 , 40

Contrasting effects of ε-ACA on plasmin formation by cell plasminogen activators

The activation of plasminogen by neuronal tPA was completely inhibited by the lysine-analogue ε-ACA (Fig. 2A), in agreement with a previous report.33 These data indicate that inhibition of plasminogen binding to the cell surface by ε-ACA prevents the formation of plasmin in situ by cells that express tPA.21 , 22 Surprisingly, cells that express uPA activate Glu-plasminogen despite the presence of ε -ACA (THP-1, Fig. 2B). Since plasmin could not be revealed at the cell surface (Fig. 2B , dotted line), our data suggest that plasminogen was activated upon contact of ε-ACA-liganded plasminogen with uPA-bearing THP-1 cells. As ε-ACA blocks both K1- and K4-LBS of plasminogen, we further explored the role of K1-LBS known to be directly implicated in plasminogen binding, using the specific mAb anti-K1-LBS, 34D3. Neutralisation of K1-LBS by mAb 34D3 impaired binding of plasminogen to the cell surface and as a consequence, cell-bound plasmin could not be detected on THP-1 cells (Fig. 2C , dotted line). However, and similarly to the effect of ε-ACA (Fig. 2B), the formation of plasmin was apparent in the supernatant despite the inhibition of plasminogen binding (Fig. 2C). These results indicate that Glu-plasminogen in complex with molecular probes that selectively block the LBS of K1 (ε-ACA or the anti-LBS-K1 mAb) adopts a conformation that could be recognized and activated by uPA-bearing cells.

Distinct effects of ε-ACA on Glu- and Lys-plasminogen activation by cellular uPA

To understand the role of conformational transitions of plasminogen (closed ⇆ open) on plasmin formation by cellular uPA, we compared the activation of equimolar amounts of either Glu- or Lys-plasminogen in the absence and presence of ε-ACA (Fig. 3). In the absence of ε-ACA, Lys-plasminogen is activated 4.5-fold faster than native Glu-plasminogen (Fig. 3A) and the amount of plasmin that remained bound to THP-1 cells shows a similar relationship (Fig. 3B). The addition of ε-ACA has no stimulating effect on Lys-plasminogen. In contrast, ε-ACA produced a 2.3-fold increase in plasmin formation from Glu-plasminogen, in agreement with data shown in Fig. 2B . The rate of Glu-plasminogen activation approaches the value obtained for Lys-plasminogen, whereas cell-bound plasmin was undetectable for both Glu- and Lys-plasminogen (Fig. 3B).

The activation experiments were then performed in presence of CpB in order to eliminate plasminogen binding and activation onto C-ter Lys residues. Our data (Fig. 3) indicate that, at the concentration of CpB used (50 μg/mL), the activation of Glu-plasminogen (Fig. 3A) at the cell surface and the amount of cell-bound plasmin (Fig. 3B) were markedly decreased. In contrast, the activation of Lys-plasminogen (Fig. 3A) was only moderately modified by CpB despite an important reduction in cell-bound plasmin (Fig. 3B) suggesting that activation at the cell membrane was practically absent. These results indicate that cell-bound uPA is able to recognize and activate Lys-plasminogen and the "Lys-like" Glu-plasminogen conformation induced by ε-ACA without requirement for molecular co-assembly on the same cell surface.

Plasmin generation via a proteolytic cell-to-matrix cross-talk

It has been generally accepted that the extended "open" conformation adopted by ε-ACAliganded Glu-plasminogen mimics plasminogen bound to lysine residues in fibrin, extracellular matrix or cells. We therefore hypothesized on the basis of the above data (Figs. 2 and 3) that Glu-plasminogen bound to fibrin, matrix proteins or cell membranes, may be recognized and activated by other cells or cell-derived microparticles present in the microenvironment and expressing uPA/uPAR complexes at their membrane. To verify this hypothesis, we analysed the activation of r-Pg-Ala^{741} , a recombinant active-site inactivated plasminogen, bound to fibrin-coated beads, by uPA-bearing EMP. The r-Pg-Ala^{741} can bind to surface carboxy-terminal lysines and be cleaved into inactive Glu-plasmin by activators.26 Therefore, if its binding to fibrin-coated beads induces a conformational change, it should be recognized and activated by uPA present on EMP. Functionality of the cross-talk was verified using native Glu-plasminogen and measuring plasmin activity. Figure 4A shows that the recognition and activation of fibrin-bound r-Pg-Ala^{741} by uPA-bearing EMP results in the formation of inactive Glu-plasmin at the surface of fibrin. Formation of Lys-plasminogen was not an intermediary step in the cross-talk mechanism, supporting the view that Glu-plasminogen bound to fibrin adopts a conformation that is recognized by EMP bearing uPA. To further verify this hypothesis, we determined the generation of plasmin activity during a matrix-to-cell cross-talk between matrix-bound native Glu-plasminogen and EMP or monocytes. Fibrin-bound plasminogen was efficiently activated, proportionally to the number of EMP incubated with the fibrin surface (Fig. 4B). In a similar manner, plasminogen-bound to the extracellular matrix protein fibronectin (Fig. 4C) and laminin (Supplemental figure 1) was efficiently activated by EMP. This activation was inhibited in the presence of amiloride or an anti-uPA specific antibody. Plasminogen bound to fibrin could also be specifically activated by uPA-bearing THP-1 cells in a cell concentration dependent manner (Fig. 4D , main graph) and by adherent monocyte-borne uPA (inset to Fig. 4D). In contrast tPA-bearing adherent neurons failed to generate plasmin (inset to Fig. 4D). Plasmin formed on fibrin surfaces resulted in fibrinolysis as indicated by the specific binding of the mAb FDP-14 directed against fibrin degradation products, in agreement with previous published data.27 In a similar fashion, the fibronectin in complex with fibrin was degraded by plasmin as detected by Western blot (data not shown).

Plasmin generation via a proteolytic cell-to-cell cross-talk

Cell-to-cell cross-talks were studied between platelets bearing plasminogen and cells bearing either uPA or tPA (monocytes and neurons, respectively) or EMP. Platelets were pre-teated with plasmin to enhance the number of plasminogen binding sites.38 After binding of plasminogen to platelets no conversion to plasmin was detected in agreement with a previous report.41 To avoid Glu-plasminogen transfer from platelets to cells bearing the plasminogen activators, monocytes and neurons were pre-treated with CpB. Under these conditions, the extent of platelet-bound Glu-plasminogen transformation into plasmin by uPA-bearing monocytes is dependent upon platelet number added until saturation (Fig. 5A). In contrast to full plasminogen activation on 1.8×10^6 platelets by monocyte-bearing uPA, plasmin formation on platelets incubated with tPA-bearing neurons was about 10% (Fig. 5A) and did not reach saturation at higher platelet concentrations. Moreover, Lys-plasminogen bound to platelets could not be activated by neuronal tPA as well (Supplemental figure 2). No plasmin was detected on monocytes or neurons surfaces indicating that plasmin was indeed generated on the platelet surface and remains associated with the platelet (not shown). Plasmin formation on platelets in absence of cells was undetectable at 1.8×10^6 plasminogen-bearing platelets (Fig. 5A , inset).

Platelet bound plasminogen could also be activated by EMP as indicated in Fig. 5B . Since EMP were pre-treated with CpB, we exclude the possibility of plasminogen exchange during the cross-talk. Plasmin was therefore formed at the surface of platelets in contact with EMP. The rate of plasmin formation during the platelet/EMP cross-talk (Vi=0.54±0.14 mOD/min) was higher than the rate of plasminogen activation (Vi=0.32±0.06 mOD/min) on EMP supplemented with an amount of plasminogen equivalent to that present on platelets (Fig. 5B). To further investigate the relevance of the intercellular cross-talk mechanism, the formation of plasmin via a platelet/monocyte cross-talk was compared to activation of plasminogen by uPA on the same cell surface (monocytes); for the purpose of demonstration, we also studied the activation of platelet-bound plasminogen by free uPA. Equivalent amounts of either plasminogen or uPA were used in all cases. The results are shown in Fig. 6A . The cross-talk mechanism resulted in a higher rate (Vi=1.1±0.26 mOD/min) of plasmin formed as compared to activation on the same cell surface (Vi=0.6±0.2 mOD/min), $p = 0.005$, thus qualifying the efficiency of the cross-talk mechanism. Addition of free uPA to platelets resulted in a non significant ($p = 0.078$) increase in plasmin formation as compared to the cross-talk. However, free uPA cannot be a relevant plasminogen activator, as most of unbound uPA in physiological

fluids is complexed to plasminogen activator inhibitor PAI-1. Specificity of the cross-talk between platelet-bound plasminogen and uPA-bearing monocytes was established by the ability of a recombinant inactive form of scuPA (r-scuPA-Gly^{159}) to inhibit the formation of plasmin (64% inhibition at 5 nM, Fig. 6B).

DISCUSSION

Specific fibrinolytic and pericellular proteolytic functions of plasmin are determined by in situ molecular co-assembly of plasminogen and its activators on cell receptors or on binding sites present on macromolecular complexes (fibrin or matrix surfaces).18 , 42 The use of tPA as a thrombolytic agent and the pericellular proteolytic activity of uPA-bearing cells are based on this paradigm.18 , 42 It has also been suggested that monocyte-uPA reduces thrombus size 43 and we have recently shown that EMP carrying uPA derived from the parent cell generate plasmin activity.19 We now show that uPA anchored on monocytes or EMP recognizes and transforms into plasmin, Glu-plasminogen bound to platelets, fibrin or extracellular matrix proteins (fibronectin, laminin). This new activation mechanism bypasses the requirement for molecular co-assembly on the same surface, via a recognition and proteolytic cross-talk pathway. Because in this plasminogen activation cross-talk, plasmin is efficiently generated on platelets or on matrix surfaces by uPA-bearing cells or MPs, it may be of potential physiological relevance in fibrinolysis or degradation of ECM components. It is worthy of note that this mechanism complies with the prerequisite for activation of plasminogen on biological surfaces, i.e. plasminogen binding to C-ter Lys residues of platelets, fibrin or ECM.18 However, it essentially differs in that the uPA is expressed on neighboring cells or MPs and in that cells bearing tPA do not reproduce this effect.

It is generally accepted that Glu-plasminogen bound to fibrin and cell surfaces adopts an open conformation similar to that induced by the C-ter Lys analogue ε-ACA.6 , 9 , 13 This conformation is called Lys-like because it is similar to the conformation of truncated Lys-plasminogen as defined by electron microscopy and small-angle neutron scattering.8 , 13 The induction of an open conformation by this lysine analogue has previously been shown and is well described in the literature (rewieved by Markus, 1996).9 On the basis of this analogy and because Lys-plasminogen, the natural open form, is activated by uPA at a higher rate than Glu-plasminogen,10 , 23 , 24 we investigated if ε-ACA-liganded Glu-plasminogen, which is unable to bind to C-ter Lys, could be activated by uPA- or tPA-bearing cells or EMP. We demonstrate that although ε-ACA-liganded Glu-plasminogen cannot bind to fibrin or cells, it was selectively activated by uPA-bearing cells. Furthermore, at equimolar concentrations, the rate of activation of Glu-plasminogen-ε-ACA complexes was higher than that of Glu-plasminogen alone, in the absence of both plasminogen binding to the cell surface and free uPA activity in the medium.

Glu-plasminogen-ε-ACA complexes were efficiently activated despite the cleavage of C-ter Lys on cells by CpB. Thus, ε -ACA-liganded Glu-plasminogen behaves as a surrogate that ensures surface-like binding conformational changes leading to its recognition and activation. Since the LBS of both K1 and K4 are simultaneously occupied by ε-ACA, we used the specific LBS-targeted 34D3 mAb to disclose the role of K1. This mAb completely inhibits plasminogen binding and activation by tPA thus underlining the role of K1 in the initial phase of plasmin formation.31 In contrast, despite the inhibition of Glu-plasminogen binding and formation of plasmin at their surface, cells bearing uPA were able to activate Gluplasminogen in the cellular microenvironment. These results indicate that uPA- but not tPAbearing cells recognize the extended “open” conformation adopted by Glu-plasminogen in complex with ε- ACA or mAb 34D3. In a similar fashion, we demonstrated in a previous study,26 that Glu-plasminogen bound to C-ter Lys residues of fibrin during ongoing fibrinolysis27 , 44 was specifically recognized and activated by scuPA26 . Furthermore, using a fibrin- or platelet-bound r-Pg-Ala^{741} in cross-talk with uPA-bearing EMP, we demonstrate that the active-site inactivated recombinant plasminogen was cleaved into inactive Glu-plasmin in situ without being transformed into Lys-plasminogen. We therefore hypothesized that Glu-plasminogen bound to C-ter Lys of cell membranes, fibrin or matrix proteins may be recognized and activated by uPA/uPAR complexes expressed on other cells or cell-derived microparticles present in the microenvironment.

First, we demonstrated that human EMP- or monocyte-borne uPA, but not tPA-bearing cells, were able to specifically activate platelet-bound Glu-plasminogen in a dose- and saturating-dependent manner. The rate of plasmin formation on platelets by monocytes was two-fold increased over the activation of plasminogen at the monocyte surfaces. The specificity of the uPA-driven proteolytic cross-talk was demonstrated by its inhibition with a recombinant form of uPA having no activator activity. The fibrinolytic cross-talk mechanism bypasses the requirement for assembly of profibrinolytic proteins on the same surface, introduces a complementary and new dimension for enhancement of fibrinolysis by platelets,41 , 45 and its efficiency suggest a potential physiological relevance. Indeed, occupancy of platelets by plasma Glu-plasminogen and a direct relation between platelet number and degree of clot lysis has been previously reported.46 ,47 Since human platelets have been shown not to express uPAR,48 these observations are in agreement with our data demonstrating a direct relationship between platelet number and plasmin generation by uPA-bearing monocytes or its derived MPs. Thus, platelet-bound plasminogen activated by monocytes or MPs bearing uPA could be an additional source of plasmin in the fibrin clot as recently suggested. 43 Our results also suggest that procedures that increase plasminogen binding to platelets may be a new direction in pharmacological enhancement of platelet fibrinolytic functions.

Second, using a similar approach, we demonstrated that Glu-plasminogen bound to fibrin surfaces, to extracellular matrix proteins (fibronectin, laminin) or to fibrin/fibronectin complexes was selectively recognized and activated into plasmin by uPA expressed on cells

or EMP. This mechanism of cross-talk may be of physiological relevance as it has recently been reported that monocytes may be involved in clot dissolution.43 ,49 Since activated monocytes and macrophages release MPs that may bear uPA, it is possible that these microparticles may participate in activation of fibrin- or platelet-bound plasminogen. Indeed, leukocyte-derived microparticles has been found in atherosclerotic plaques,50 where they can initiate fibrinolytic or proteolytic activities that may destabilize the atheroma plaque. A similar interaction may take place during inflammatory processes where primed cells could initiate a proteolytic cross-talk with plasminogen bound to other cells or to the matrix as suggested in the proposed model (Fig. 7).

Altogether, these data provide a mechanistic support to the proteolytic cross-talk mechanism described above and suggest that, in inflammatory states or in cancer, plasminogen bound to platelets, fibrin, extracellular matrices or adherent cells could be efficiently activated by uPA expressed on migrating cells or cellular microparticles. For instance, plasminogen bound to cells may be transported into non-easily accessible tissues like the brain and be activated in situ by resident or migrating glial cells expressing uPA.51

In conclusion , we propose a new mechanism for plasmin formation that not only does bypass the requirement for co-assembly of plasminogen and uPA on the same surface but it also makes Lys-plasminogen dispensable. These heterotypic cell-to-cell (platelets/monocytes; platelets/MP), cell-to-matrix (THP-1/fibrin) or microparticles-to-matrix (MP/fibrin) proteolytic cross-talk represents an alternative pathway for localized plasmin formation that may be relevant to processes implicating cell migration and microparticle dissemination i.e . inflammation or angiogenesis. Finally, our data provide additional evidence for a novel role of microparticles and platelets, as vectors that generate and propagate plasmin fibrinolytic and/or proteolytic activity, and could thereby constitute a pharmacological tool.

Ackowledgements:

These studies were supported by funds from the Inserm (Institut National de la Santé et de la Recherche Médicale) and the Lower-Normandy Regional Council. EAC, LD and LP are members of the European Community's Seventh Framework Programme (FP7/2007-2013) under grant agreement n° 201024. The Center for Molecular and Vascular Biology is supported by the Excellentie financiering, KU Leuven. T.D. is a recipient of a PhD fellowship awarded by the Nouvelle Societé Française d'Athérosclérose. L.D. is a recipient of a PhD fellowship from the Education and Research French Ministry.

Footnotes:

Disclosure statement: the authors declare no competing financial interests.

Authorship T.D. and L.D. performed research, analyzed data and wrote the manuscript. R.L. performed research and participated in manuscript drafting. L.P. provided experimental support and participated in manuscript drafting. F.D-G. analyzed data and participated in manuscript drafting. H.R.L. contributed analytical tools and participated in manuscript drafting. E.A-C. designed the research, analyzed data and wrote the manuscript.

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Figure 1

Identification of activators and cellular activation of plasminogen

A. Fibrin autography of platelets, cortical neurons, HMEC-1 and THP-1 cells. The samples were electrophoresed on 8% (w/v) polyacrylamide, SDS was then exchanged with 2.5% (w/v) Triton X-100 and the gel overlaid on a fibrin-agar indicator gel. The picture was taken after 4 h at 37 °C. The position of purified controls (Pn: plasmin, tPA and uPA) is indicated on top. The thin vertical line indicates assembly from the same gel. The thick vertical line separates two different gels. **B.** THP-1 cells (10^5 cells/well) were incubated with varying concentrations of plasminogen (0 to 3 μM) and 0.75 mM CBS0065. Kinetics of plasmin formation (mOD/min) was followed by measuring the realease of p -nitroaniline. Data were fitted according to the Michaelis–Menten equation (Km = 492 nM).

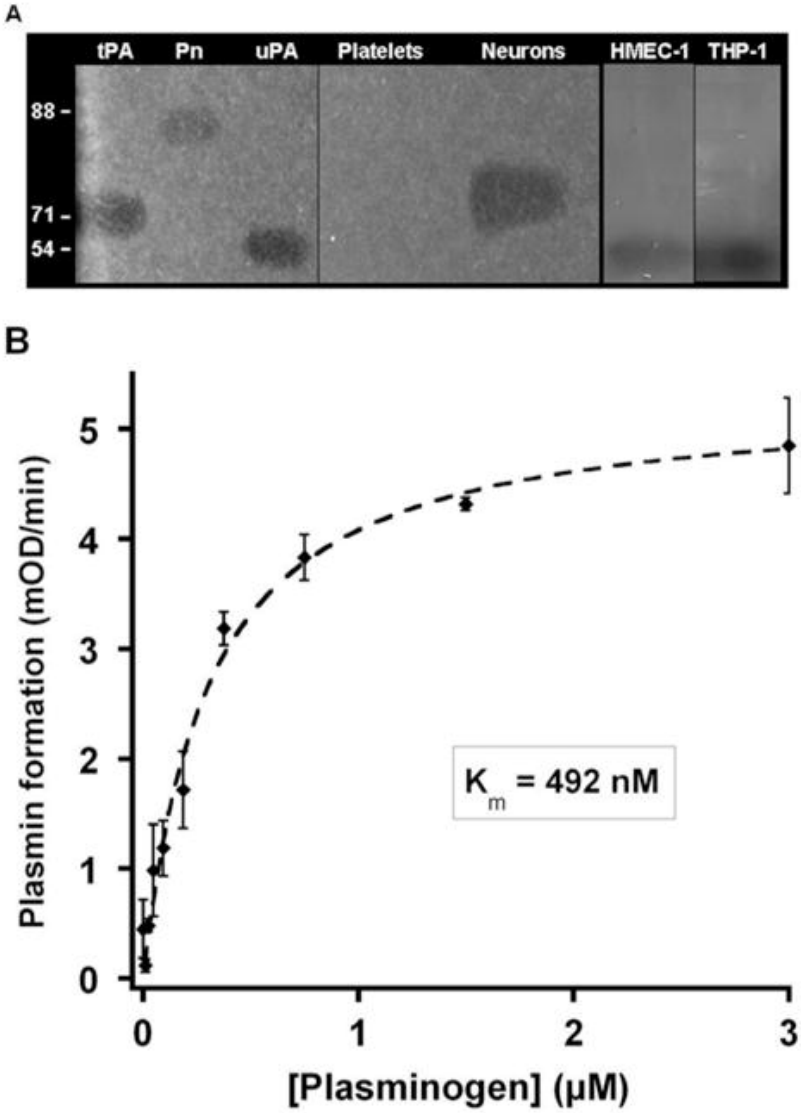


Figure 2

Cellular activation of plasminogen: effect of LBS ligands

A. Cortical neurons (10^5 cells/well) and **B** . THP-1 cells (10^5 /well), were incubated with 125 nM Glu-plasminogen supplemented with varying concentrations of ε-ACA (0 to 25 mM) and 0.75 mM CBS0065. Plasmin formation (•) was detected by measuring the release of p-nitroaniline. **C** . THP-1 cells (10^5 /well) were incubated with 125 nM Glu-plasminogen supplemented with of 0 to 1 μM anti-K1-LBS mAb 34D3 and 0.75 mM CBS0065. A, B, C. After detection of plasmin formation, the cells were washed twice with PBS and incubated with 0.75 mM CBS0065 to detect cell-associated plasmin (□). Results are expressed as a percentage (mean ± SD, n = 3) of plasmin formation or of cell-associated plasmin activity in the absence of ε-ACA or mAb.

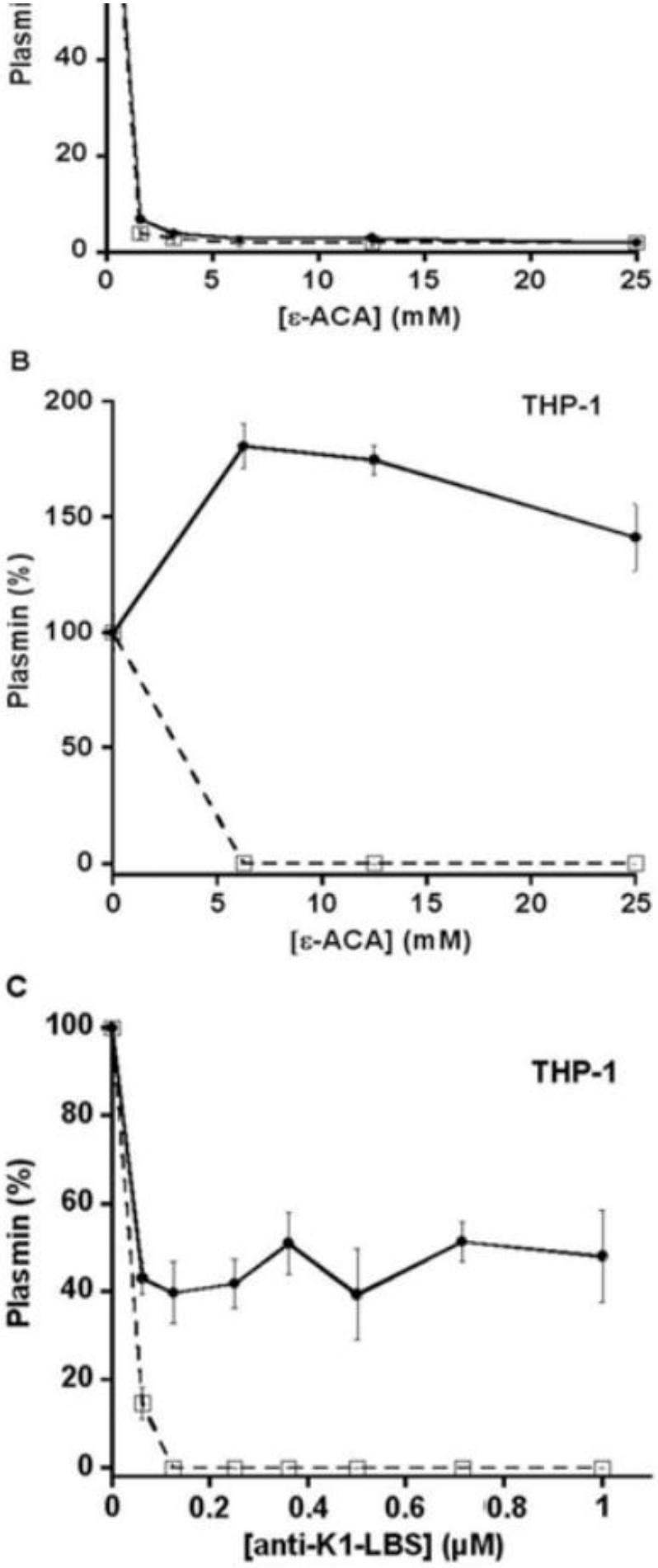

Plasmi
40
20
0
0
5
10
15
20
25
[ε-ACA] (mM)
B
THP-1
200
150
100
50
0
Plasmin (%)
0
5
10
15
20
25
[ε-ACA] (mM)
C
THP-1
100
80
60
40
20
0
Plasmin (%)
0
0.2
0.4
0.6
0.8
1
[anti-K1-LBS] (μM)

Figure 3

Effect of ε-ACA and carboxipeptidase B on Lys- and Glu-plasminogen activation by cellular uPA

THP-1 cells (10^5 /well) were incubated with 500 nM Lys- (grey bars) or Glu-plasminogen (open bars) in medium alone or supplemented with ε-ACA (5 or 25 mM) and 0.75 mM CBS0065. Carboxypeptidase B, CpB (50 μg/mL), pre-treated THP-1 cells were incubated with plasminogen and CBS0065. Rate of plasmin formation (**A**) and amount of cell-associated plasmin (**B**) were detected as indicated in figure 2 . Bars represent the amount of plasmin formed or associated to the cells (mOD/min) versus the concentration of ε-ACA or after CpB treatment (mean ± SEM, n = 3). Stars indicated significant changes as compare to Lys-Pg (*$p < 0.05$)/Glu-Pg (§ $p < 0.05$) alone.

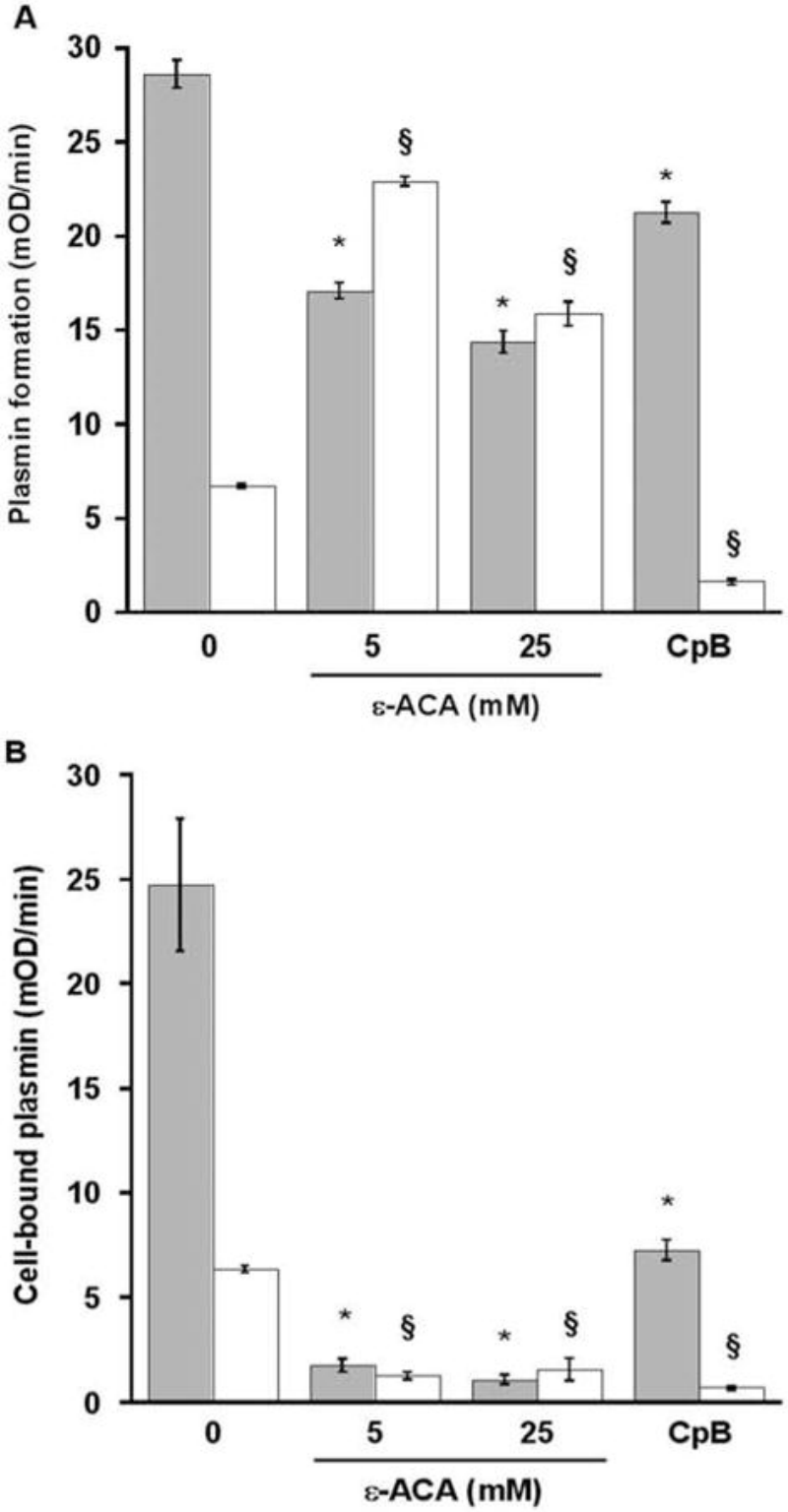


Figure 4

Proteolytic cross-talk: activation of fibrin- and fibronectin-bound plasminogen by cellular microparticles

A. Native or recombinant active-site inactivated Glu-plasminogen (Glu-Pg, r-Pg-Ala^{741}) at 1 μM were bound to fibrin-coated beads for 1h at 37°C. Fibrin-coated beads with bound plasminogen were then incubated with 10^6 endothelial microparticles (EMP) in a final volume of 200 μ L. After overnight incubation at 22°C, the fibrin-coated beads were sedimented by centrifugation and resuspended in 10 mM Tris-HCL pH 6.8 containing 10 % SDS to elute fibrin-bound plasminogen derivatives. The supernatant was electrophoresed under non-reducing conditions, proteins were transferred to PVDF membranes and revealed with a HRP-conjugated mAb (150 ng/mL) directed against plasminogen K1. The Western blot shows Glu-plasmin formaton by EMP. Purified plasmin is shown as reference. **B, C.** Glu-plasminogen (1 μM) was bound to fibrin (**B**) or fibronectin (**C**) surfaces. After 3 washes with PBS, EMP were added at varying concentrations. **D** . Glu-Plasminogen (1 μM) was bound to fibrin surfaces (main graph) or to fibrin-coated beads (inset). THP-1 cells were then added to fibrin surfaces at varying concentrations (main graph) and 2.5×10^5 fibrin-coated beads were incubated with 10^5 adherent monocytes or neurons (**inset**). The formation of plasmin was detected by measuring the release of p -nitroaniline from the chromogenic substrate CBS0065 added at 0.75 mM. Bars represent the amount of plasmin formed (mOD/min, mean ± SEM, n = 3) by THP-1 cells on fibrin (**B, D)** and fibronectin (**C**), and by adherent monocytes or neurons on fibrin-coated beads (**Inset to D**). Amil: amiloride, anti-uPA, IgG: antibody against uPA and its non-immune IgG control. Stars indicated significant changes as compare to activation without THP-1 (**A**) or EMP (**B, C**) or activation on neurons (*$p < 0.05$); § indicated changes with inhibitors as compare to activation at 5×10^5 EMP (§ $p < 0.05$).

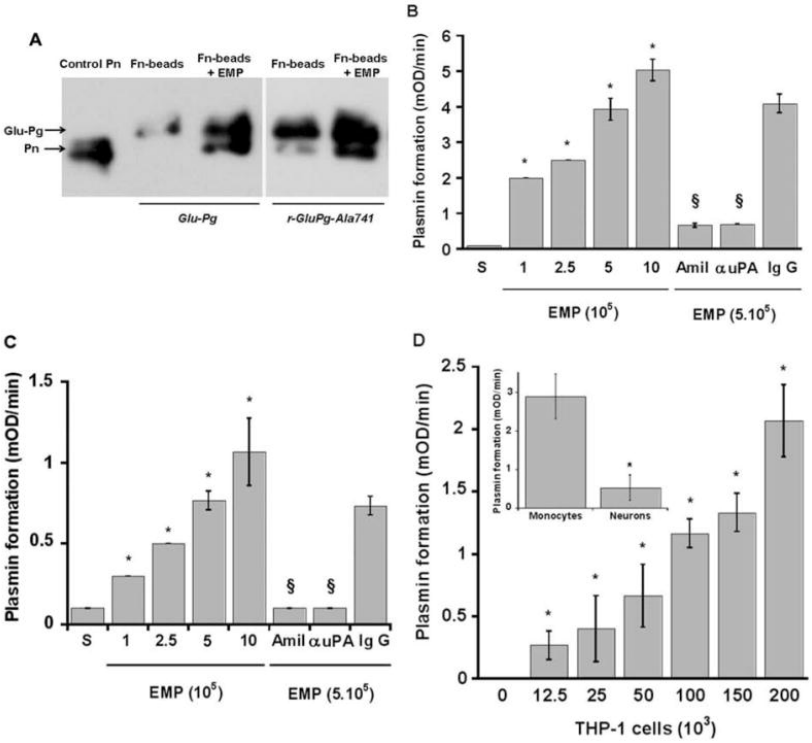


Figure 5

Proteolytic cross-talk: activation of platelet-bound plasminogen by cells bearing uPA (monocytes) or tPA (neurons)

Glu-plasminogen (1 μM) was bound to platelets as indicated in Methods. **A.** After treatment with 50 μg/ml CpB, monocytes (●) or neurons (■) were incubated with plasminogen-bearing platelets at varying concentrations (0 to 1.510^6 /well) in the presence of 0.75 mM CBS0065. The formation of plasmin (mOD/min) was detected by measuring the release of p -nitroaniline. **Inset.** Detection of plasmin formation on platelets (1.8×10^6) in the absence of cells or incubated with monocytes or neurons (10^5 cells). Results are expressed as rate of plasmin formation (mean ± SEM, n = 3). **B.** Glu plasminogen (1 μM) was bound to platelets as indicated in Methods. After treatment with 50 μg/ml CpB, endothelial microparticles (EMP) were incubated with 5×10^6 platelets bearing plasminogen (●) or with 3 nM plasminogen (concentration equivalent to that bound to platelets), or with buffer (▲) in the presence of 0.75 mM CBS0065. The formation of plasmin (mOD/min) was detected by measuring the release of p- nitroaniline. Results are expressed as rate of plasmin formation (n = 3). A representative experiment is shown.

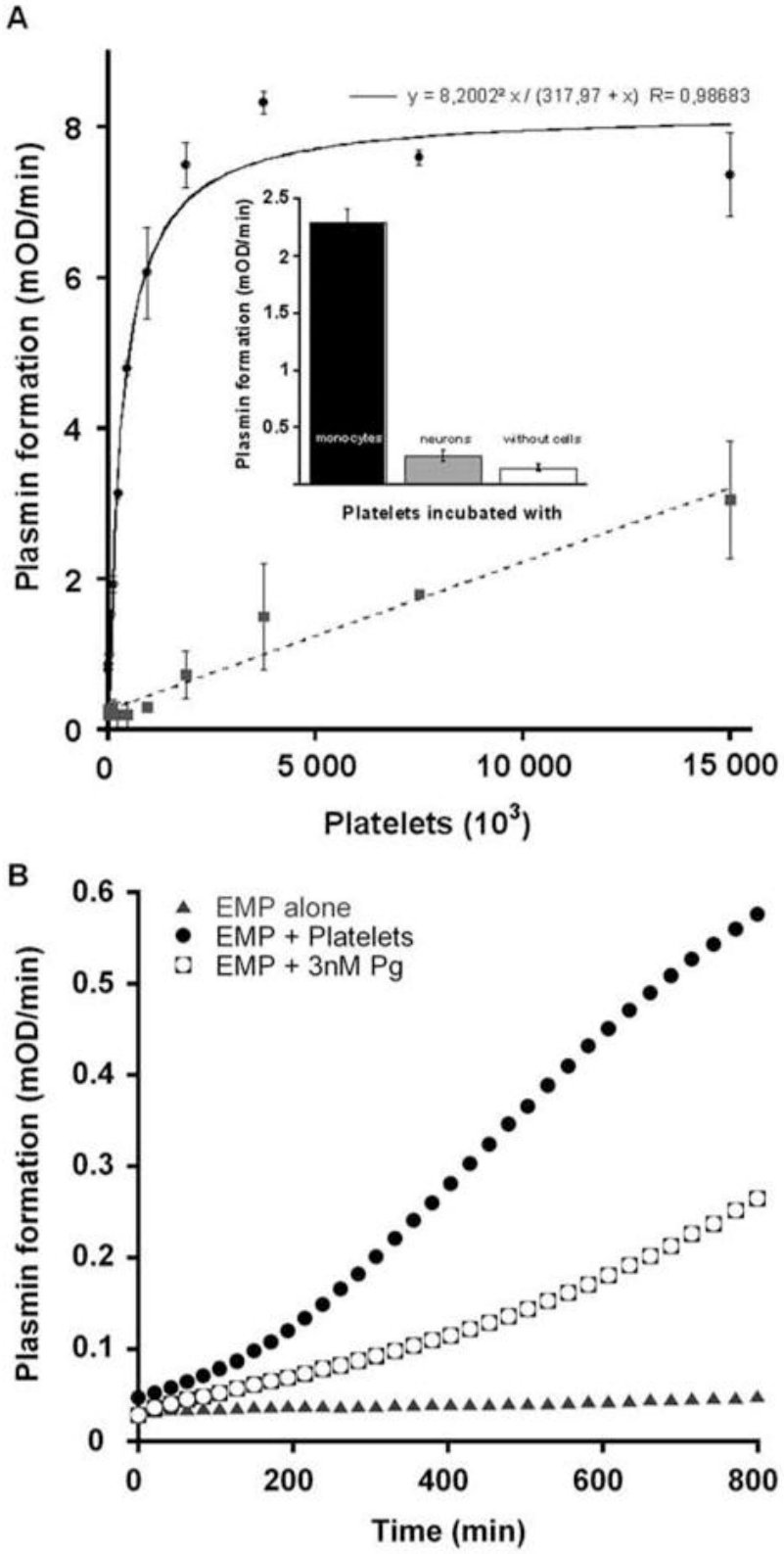


Figure 6

Efficiency and specificity of the plasminogen cross-talk

Glu-plasminogen (1 μM) was bound to platelets as indicated in Methods. **A.** After treatment with 50 μg/mL CpB, adherent monocytes were incubated with 5×10^6 platelets bearing plasminogen (cross-talk) or with plasminogen (concentration equivalent to plasminogen bound to platelets) (same surface) in the presence of 0.75 mM CBS0065. In a parallel experiment, uPA (concentration equivalent to uPA bound to monocytes) was incubated with 5×10^6 platelets bearing plasminogen (free uPA) in the presence of 0.75 mM CBS0065. Results are expressed as rate of plasmin formation (mean ± SEM, n = 3, triplicates). NS: non significant (p = 0.078) *p <0.005, # p<0.014. **B.** Monocytes were incubated with 1 nM of native uPA and varying concentrations of a non-active mutant uPA (r-scuPA-Gly^{159}). Monocytes were then incubated with 5×10^6 platelets bearing plasminogen, in the presence of 0.75 mM CBS0065. The formation of plasmin (mOD/min) was detected by measuring the release of p -nitroaniline (mean ± SEM, n = 2, triplicates). A representative experiment is shown (p = 0.023, 0 vs 5 nM r-scuPA-Gly^{159}).

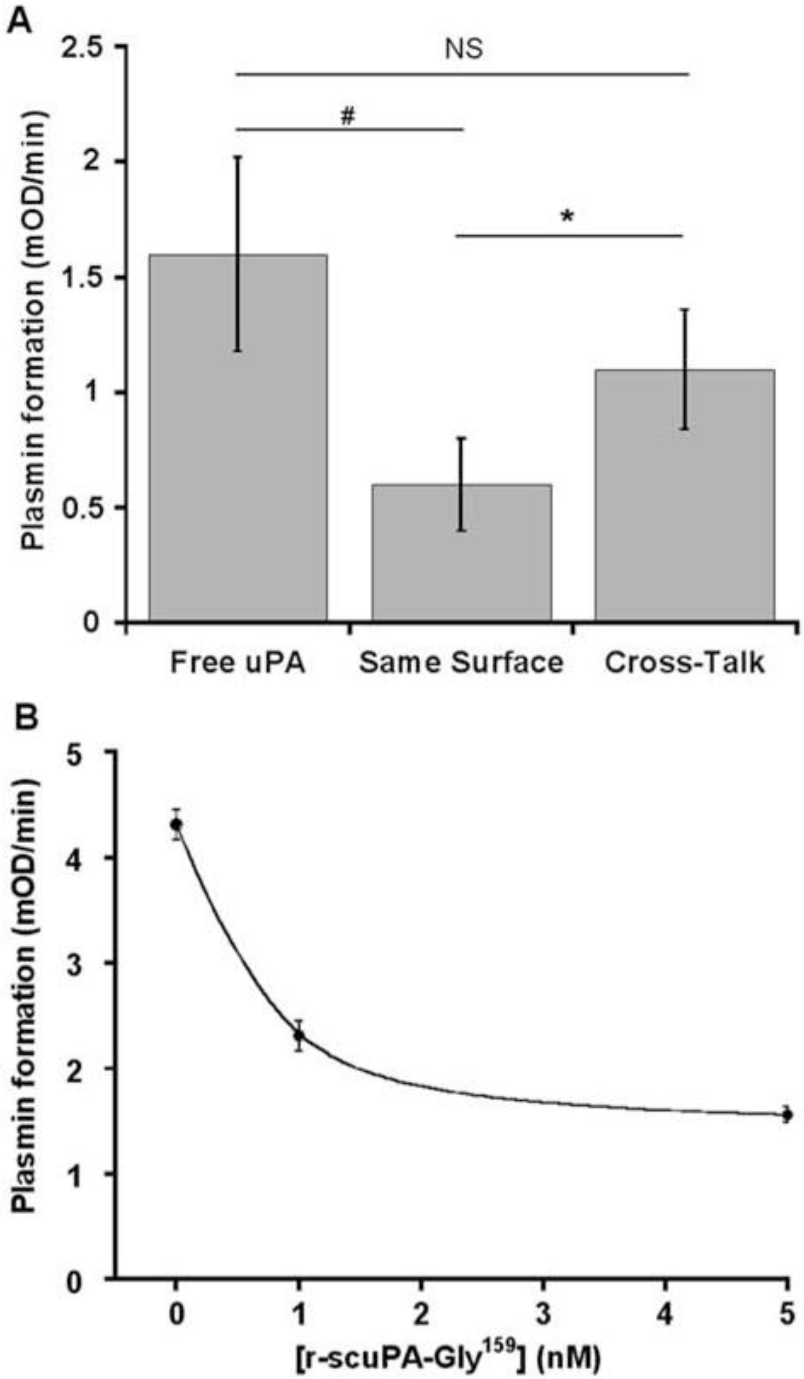
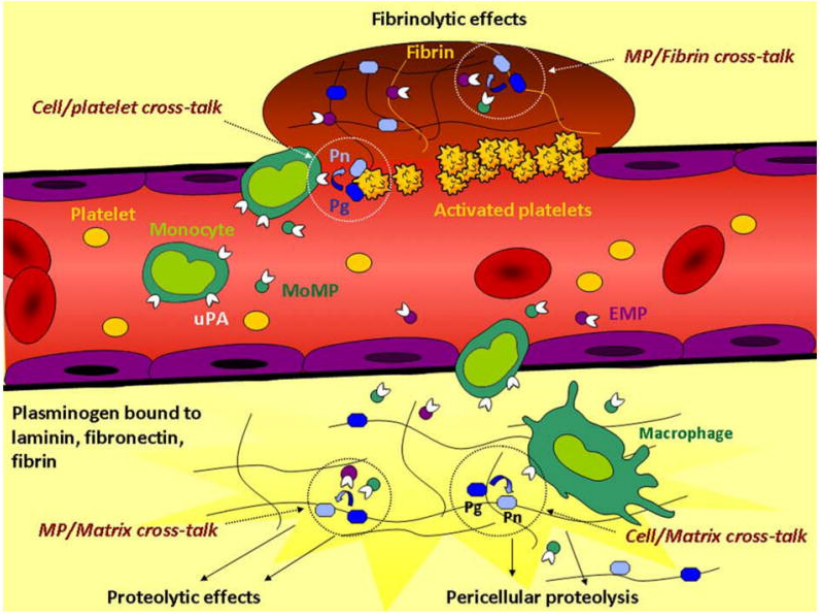


Figure 7

Model of fibrinolytic and proteolytic cross-talk

In inflammatory processes of the vascular wall, activation of coagulation leads to fibrin deposits. Within this setting and according to data presented here, the fibrinolytic cross-talk mechanism could be an intermediary pathway for fibrinolysis and pericellular proteolysis. Fibrinolytic effects (upper part): plasminogen (Pg) bound to fibrin or to platelets could be activated by either monocyte- or microparticle-borne uPA (EMP or monocyte-derived microparticles, MoMP). In extracellular matrix (ECM) remodeling in the vascular wall (lower part), plasminogen bound to matrix components could be activated by microparticle-borne uPA (EMP or MoMP) or by macrophage-borne uPA.