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Plasmin on adherent cells: from microvesiculation to apoptosis

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SYNOPSIS

Cell activation by stressors is characterised by a sequence of detectable phenotypic cell changes. The strength of a given stimulus induces modifications in the activity of membrane phospholipids transporters and calpains, which leads to phosphatidylserine exposure, membrane blebbing and the release of microparticles(nanoscale membrane vesicles).This vesiculation could be considered as a warning signal that may be followed, if the stimulus is maintained, by cell detachment-inducedapoptosis.
In this study, plasminogenincubated ontoadherent cells is activated into plasmin by constitutively expressed tPA or uPA. Plasmin formed on the cellular membrane then induces an unique response characterized by membrane blebbing and vesiculation.Hitherto unknown for plasmin, these membrane changes are similar to those induced by thrombin on platelets. If plasmin formation evolves, matrix proteins are then degraded, cells loseattachment and enter the apoptotic process, characterized by DNA fragmentation and electron microscopy features.
This sequence of events was experimentally documented atall these stages. Since other proteolytic or inflammatory stimuli may evoke similar responses by distinct adherent cells, this sequence can be applied to distinguish activated adherent cells from cells entering the apoptotic process.This is a major definition crucial to the identification of mediators, inhibitors and potential therapeutic agents.

Key words: Plasminogen, membrane blebbing, microparticles, apoptosis, electron microscopy

Abbreviations used:
Microparticles: MPs
Urokinase type plasminogen activator: uPA
Tissue-type plasminogen activator: tPA
Extracellular matrix: ECM
ε-aminocaproic acid: ε-ACA
Tetrazolium salt 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide: MTT
Carboxypeptidase B: CpB
4’, 6-diamino-2-phenylindole: DAPI
Terminal Deoxynucleotidyltransferase-mediated dUTP Nick End Labeling: TUNEL

INTRODUCTION

Cell response to a number of stressors and inflammatory mediators iskey to maintenance of tissue homeostasis. Initial cell activation may evolve to apoptosis depending on the type and intensity of stimuli [1]. Early manifestations of this cell activation process are the formation of membrane blebs and the shedding of nanoscale membrane fragments known and designated hereafter as microparticles(MPs, 0.1 to 1 µm) [2]. This phenomenon is also an early event of cells entering apoptosis. Since the first discovery of MPs in a platelet-free plasma [3, 4], the most well known cellular MPs are those of platelet, leukocyte, erythrocyte and endothelial cell origin found in circulating blood [5]. A number of studies have demonstrated that stimulation of these cells is followed by the characteristic features of cell activation: increased levels of cytoplasmic calcium associated to exposure of phosphatidylserine and activation of calpains (EC 3.4.22)[6]. The increase in intracellular calcium induces a disordered state in the concerted activity of membrane transporter proteins (flippases, floppase and scramblases) that maintain the phospholipids asymmetry of quiescent cells[7, 8]. As a result, the procoagulant phosphatidylserine is translocated from the inner leaflet to the external leaflet of the membrane. The activated calpains cleave cytoskeleton filaments and thereby facilitate membrane blebbing and shedding of MPs. In addition to their procoagulant activity, MPs carry at their surface identity antigens that characterize their cellular origin. Since they also convey proteolytic glycoproteins, growth factor or inflammatory mediators, they are currently considered as a storage pool of bioactive effectors [9]. The proteolytic mediators that have been identified include the plasminogen activation system and the matrix metalloproteases(EC 3.4.24) [10-13].

The plasminogen activation system is involved in fibrinolysis and in pericellular proteolysis [14]. Finely regulated, cellular plasminogen activation plays an essential role in extracellular matrix (ECM) remodelling[1], cell migration [15] and survival [16].The central mechanism is the transformation of plasminogen into plasmin(EC 3.4.21.7), the active enzyme, at cell membranes and ECM or atthe surface of fibrin, by the urokinase- or the tissue-type-plasminogen activator, uPA (EC 3.4.21.73) or tPA (EC 3.4.21.68), respectively. MPs function as a catalytic surface for plasmin formation by providing both plasminogen activators and plasminogen binding sites[10]. Interestingly, the uPA system of MPs also participates in the formation of plasmin at the surface of platelets, fibrin or the ECM *via* a fibrinolytic/proteolytic cross-talk mechanism recently described [17]. Thrombin (EC 3.4.21.6), another serine protease, is known to induce the release of MPs by platelets, but the plasmin potential to induce vesiculation is not known as yet.

Beyond activation-dependant release of MPs, the survival of cells within structural-functional units involving tissue specific components and the microvasculature (e.g. neurovascular unit, glomerulus and pulmonary alveolus), depends on dynamic cell-matrix interactions that ensure their adhesion to the substratum and tissue cohesion. Thus, in the absence of any ECM interaction, human endothelial cells rapidly enter apoptosis [18]. Accordingly, excessive proteolysis of the ECM by cells that express a plasminogen activator system results in loss of cell anchorage and apoptosis [19], a phenomenon that may be of relevance in pathological situations *in vivo*[20]. Discerning the steps of this cell activation that may induce apoptosis is therefore a key to identify mediators, inhibitors and potential therapeutic targets.

We unveil in this report that plasmin provokes the release of cell-derived MPsand proposea sequence of events initiated byplasminogen activation on adherent cellsand spanning from early blebbing and vesiculationto subsequent cell detachment and apoptosis/survival.Because other proteolytic or inflammatory stimuli may evoke similar responses by distinct adherent cells, this mechanisticproceduremaybe applied to distinguish activated adherent cells from cells entering the apoptotic process.

EXPERIMENTAL

Reagents and proteins

Human Glu-plasminogen used in this study waspurified and characterized as described [21] and was over 99% pure as assessed by SDS-PAGE and by amino-terminal sequence analysis. Plasmin was prepared by activation of Glu-plasminogen with immobilized uPA according toWiman and Wallen[22]with modifications. Rabbit anti-mouselaminin polyclonal antibodywas kindly provided by H. P. Erickson (Duke University Medical Center, Durham, NC). The peroxidase-labelled monoclonal antibody directed against plasminogen kringle1 (CPL15-PO) was prepared as described [19]. Active-site blocked plasmin (D-Val-Phe-Lys-chloromethylketone, dihydrochloride, Pn-VFK) was prepared as described previously [16]. uPA(140 000iu/mg) and tPA (578 000iu/mg) (both over 99% single-chain form) were obtained from Biopool AB (Umea, Sweden) and Abbot (Chicago, Il, USA), respectively.Aprotinin was a kind gift from Bayer HealthCare AG (Leverkusen, Germany). Glutamine, foetal calf serum and DMEM-Ham's F-12 medium wereobtained from Invitrogen (Carlsbad, CA, USA). The chromogenic substrate selective for plasmin (methylmalonyl)-hydroxyprolylarginine-*para*-nitroaniline (CBS0065) was from Stago (Asnieres, France). The plasmin inhibitor VFK was from calbiochem (Darmstadt, Germany). The lysine analogue ε-aminocaproic acid (ε-ACA), the tetrazolium salt 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), dimethylsulfoxide (DMSO), carboxypeptidase B (CpB, porcine pancreas),4’, 6-diamino-2-phenylindole (DAPI) were from Sigma (St-Louis, MO, USA). The terminal Deoxynucleotidyltransferase-mediated dUTP Nick End Labeling (TUNEL) was from Roche Applied Science (Neuilly sur Seine, France).

Identification of cell plasminogen activators

Chinese hamster ovary cells (CHO-K1 cells, American Type Culture Collection CCL-61, fibroblast phenotype), human embryonic kidney cells (HEK-293, [23]) and human microvascular endothelial cells (HMEC-1, [24]) were used as prototype adherent cells. Cells were grown in a 37°C humidified atmosphere of 5% CO_2 using DMEM-Ham's F-12 medium (CHO-K1 cells) or RPMI medium (HEK-293 and HMEC-1 cells) supplemented with 2 mM L-glutamine and 10% foetal calf serum. Cells were then seeded in 96 or 24 wells plates, depending on experiments. Fibrin autography following SDS-PAGE was performed as described previously [25]. Briefly, cells were lysed in 100 mMTris-HCl buffer, pH 8.1, containing 1% Triton X-100. Proteins in cell lysates (100 µg) and reference proteins (10 µL of tPA 5 nM, uPA 1 nM and plasmin 500 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 gel containing 1 mg/mL of bovine fibrinogen, 100 nM 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).To verify the activator identity, cell lysates were incubated with a polyclonal antibody directed against tPA or uPA and their residual activity detected by radial diffusion on fibrin-agarose gels.

Cell activation by plasmin

Cells were incubated with Glu-plasminogen (1µM) at 37°C, in the presence of 0.75 mM of CBS0065, a plasmin selective chromogenic substrate. In parallel experiments, the effect of various inhibitors (ε-ACA, a lysine analogue that blocks plasminogen binding to cells; CpB,

an exopeptidase that cleaves C-ter Lys residues on cells; aprotinin, a serine-protease inhibitor with restricted specificity) on plasminogen activation (1 µM) was determined in the presence of 0.75 mM of CBS0065.
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 slopes of A_{405nm}*versus* time. Initial rates were computer-calculated at the inflexion point of the curve by non-linear least square analysis, and were fit to the michaelis-menten equation considering a non-specific component proportional to plasminogen concentration.

$$\frac{V_{\max}[Pg]}{Km + [Pg]} + k[Pg]$$

Detection of cell blebbing and release of microparticles by activated cells
Supernatants of activated cells,collected at different time points during plasminogen activation, were supplemented with 2% glutaraldehyde to fix detached cells beforecytospinning at 800 g for 5 min (cytospin®Thermo Fisher Scientific, USA) on small Thermanox® (NUNC, USA) coverslips. These coverslips support cells all along the preparation until the end of polymerization and embedding of the resin for electron microscopy. Rapidly after cytospinning, the Thermanoxcoverslips were transferred into a cap containing phosphate buffer. Cells were then rinsed 3 times in phosphate buffer (0.1M, pH 7.4) before and after post-fixation with 0.5% osmium tetroxide, dehydrated through a graded ethanol series (70% 2x, 95% 3x, 100% 4x) and finally embedded in epon resin. After polymerization, the coverslips were removed and ultrathin sections were stained with 2% uranyl acetate and lead citrate. All observations were performedwith a JEOL 1011 transmission electron microscope. Images were acquired with a megaview III camera (SIS).

Microparticle isolation
MPs were isolated from culture medium conditioned by subconfluentadherent cells stimulated for 24 h with 1 µM plasminogen. Culture supernatants were collected and cleared from detached cells or large cell fragments by centrifugation at 1500 g for 15 min. The supernatants were then centrifuged at 20000*g* for 90 min at 4°C. PelletedMPs were washed twice using the same conditions and were suspended in phosphate-buffered saline.

Determination of cell survival by the MTT method
At indicated time intervalsduring plasminogen activation, the conditioned medium was carefully removed from a 96-well plate without disturbing the cell monolayer and replaced with 100 µL of 0.5 mg/mLMTT. The tetrazolium salt was transformed into formazan by the mitochondrial succinatedeshydrogenase of living cells. After 1 h at 37°C, excess MTT was removed and the formed crystals of formazan were dissolved in 100 µL of DMSO and colorimetrically detected at $A_{550\ nm}$. Absorbance readings are proportional to the number of living cells. The results are expressed as a percentage compared to control cells.

TUNEL and DAPI staining
After cytospinning of cell supernatants collected at different time intervalsof plasminogen activation, TUNEL was used to visualize DNA fragmentation. The cells were then counterstained with DAPI to visualize all nuclei. After washing, the slides were mounted and observed under an epifluorescence microscope. The TUNEL index was calculated as the percentage of TUNEL-positive nuclei relative to total DAPI-stained nuclei.

Western Blot

Plasmin-activated cellsor MPswere lysed in 100 mMTris-HCl buffer, pH 8.1, containing 1% Triton X-100. Proteins in lysates (10 µg) were electrophoresed in an 8% polyacrylamide gel under reducing conditions. Proteins were transferred to PVDF membranes and revealed with primary specific antibodies (a sheep antibody directed against uPA, a rabbit antibody directed against laminin and a peroxidase-labelled monoclonal antibody directed against plasminogen kringle1)at proper concentrations and HRP-coupled secondary antibodies when indicated.

Statistical analyses

Data are expressed as mean ± standard error of the mean (SEM). The statistics were performed using nonparametric tests of Kruskall Wallis and of Mann Whitney (MYSTAT12 software). Statistical significance was set at $p < 0.05$.

RESULTS-DISCUSSION

Cell membrane stability and functioning depend on coupling of the phospholipid asymmetric transbilayer to the cytoskeleton. In adherent cells, stability is further enhancedby coupling of integral membrane protein receptors (integrins) to ECM glycoproteins such as fibronectin, laminin, collagen, thrombospondins and tenascins that act as a scaffold surface for cell adhesion. They function as a mediator of cytoskeleton reorganization leading to formation of focal adhesions and intracellular signalling[26, 27] that direct cell growth, morphology, migration and anchorage-dependent survival [28]. Disruption of the transbilayerphospholipid asymmetry or disruption of ECM-integrin interactions by proteolysis or integrin antagonists for example, is associated with cell activation or pathologic conditions. Since they may have consequences on membrane cytoskeleton reorganization and on cell adhesion during tissue remodeling or in pathological proteolysis [20, 29, 30], we explored the possibility that plasmin formation on cells may induce these phenotypic cell changes. We provide here the first demonstration that plasmin generated at the surface of adherent cells induces an early response characterized by membrane blebbing and vesiculation, i.e., release of MPs. In addition, we show that plasmin proteolyses ECM proteins and thereby disruptinteractionswith cells that result in phenotypic changes characteristic of cell detachment and apoptosis. Thus, in agreement with previous reports [8, 31], localized decoupling of the cytoskeleton to the membrane results in formation of a bleb exposing phosphatidylserine [8] and loss of anchoring to the matrix results in reorganization of focal adhesion points and cell detachment [31]. Membrane blebs, MPs and cells activated or entering apoptosis express phosphatidylserine at the outer leaflet [6]. Fluorescent annexin A5 has been used to detect the exposed phosphatidylserine by flow cytometric analysis [32]. Since MPs may contaminate cell preparations [33], the annexin A5 binding assay cannot discriminate amongst cells or MPs expressing phosphatidylserine [34]. To better identify early morphological changes (membrane blebbing and vesiculation) before anchorage-depending survival of cells is compromised, we first determined the ability of cells to express plasminogen activators and then monitored plasmin formation and its effects on cell blebbing and survival.

We demonstrate that CHO-K1 and HEK-293 cells express active tPA whereas HMEC-1 cells express uPA as indicated by the position of a fibrinolytic band similar to the migration of purified uPA or tPA detected by fibrin zymography (Fig. 1A, upper panel). Confirmation was obtained by inhibition of the fibrinolytic activity with specific antibodies as detected by radial diffusion on fibrin-agarose gels(Fig. 1A, lower panel). Unrelated antibodies had no effect on plasminogen activation(Fig. 1A, lower panel). Kinetics of plasmin formation (Fig. 1B) indicated that plasminogen incubated with cells were assembled at their surface and

transformed into plasmin in a time-, lysine- and dose-dependent manner until saturation (Fig.1C). Kinetic parameters were determined by fitting data to the Michaelis-Menten equation (Vmax [Pg] / (Km+[Pg])) + k[Pg]); Table 1). The calculated Km value is comparable to values obtained for tPA expressed by other cell types including endothelial cells[35] and neurons[16]. It is also comparable to values determined for the activation of plasminogen by tPA on fibrin (40 to 160 nM[36, 37],suggesting that the cell surface providesa similar plasminogen activation enhancement. Lysine-dependant specific plasminogen binding was indicated by inhibition of plasmin formation both with the lysine analogue ε-ACA that blocks the LBS of plasminogen, and with CpB that cleaves C-ter lysine residues from the cell surface. The activity of plasmin formed *in situ* was efficiently inhibited by aprotinin (Fig. 1D).

Since thrombin, a serine protease like plasmin, induces membrane blebbing and platelet vesiculation, we explored the possibility that plasmin may induce a similar response on adherent cells. Membrane blebbing is a short-lived phenomenon observed at the very initial phase of cell stimulation that can only be visualized by electron microscopy. For that purpose, the early response of cells to plasmin formation was interrupted by glutaraldehyde fixation, thus allowing capture of multiple blebbing of the membrane and formation of numerous MPs that were about to be released by cells (Fig. 2). A small number of MPs could be detected in culture control conditions (Fig. 2A, Table 1). After incubation of cells with plasminogen, the number of MPs detected in the culture medium was dramatically increased, independently of the adherent cell type (Fig 2A). These MPs are around 300 nm in size and contain substructures having an electron density similar to that of cell cytoplasm, which are surrounded by a well-defined membrane (Fig 2B,C, D). This phenomenon was dependent on the activity of plasmin as indicated by its prevention in the presence of aprotinin (data not shown). Since plasmin was the onlyaprotinin-sensitive serine protease present in the culture supernatant (Fig. 1B), these results indicate that membrane blebbing is one of the early responses of cells to plasmin.MPs, that are released, carry the plasminogen activator of their parental cell (e.g. uPA for HMEC-1, Fig 3A) at their surface and plasmin(ogen) bound at the membrane (Fig 3B). These results confirm that MPs could be considered as potential messengers of proteolytic activity [9, 10].

As indicated above, the first and immediate consequence of cellular stimulation by plasmin is membrane blebbing followed by the release of MPs. The continuous formation of plasmin has other consequences on cell phenotype and behaviour, which are related to the proteolytic activity of plasmin on the substratum. Active plasmin proteolyses and degrades components of the ECM including laminin (Fig. 3C) and fibronectin[38]. In the absence of a substratum for anchorage, the cells became round (Fig. 4A) and gradually detached from their support as shown by the amount of residual living cells (MTT assay, Fig. 4B). After 48 h of incubation with plasminogen, the detached CHO-K1 cells entered the apoptotic process as suggested by DNA fragmentation observable by TUNEL (Fig. 4C, D).
Electron microscopy allowed detailed observation of cell morphology and characteristic apoptotic changes (Fig. 5). In control cells, the nucleus membrane is clearly delimitated and the characteristics of normal cytoplasm are maintained including endoplasmic reticulum and mitochondria with their internal crests (Lower panel, Fig. 5A). In plasminogen-treated cells (Fig. 5B; 48 h), the nucleus shows chromatin condensation, the cytoplasm is disorganized, contains lysis vesicles and mitochondria become electron dense (Lower panel, Fig 5B). After 72 h of stimulation, consequences of plasmin formation are dramatic: higher condensation and fragmentation of the chromatin (Fig. 5C).

To investigate the relation between plasmin formation and cell detachment, we used active-site blocked plasmin, Pn-VFK (Fig.6A). As a consequence, Pn-VFK is unable to develop a proteolytic activity on ECM components, e.g. laminin (Fig. 3C). In the absence of ECM degradation, cells do not detach from their support and remain viable (Fig.6B).

Altogether, these data indicate that upon plasmin formation, the proteolytic activity of this enzyme induces (1) at the cell level, membrane blebbing and vesiculation and (2) on matrix proteins, cell detachment-induced apoptosis.
Conditioned medium from cell culture experiments and animal or human biological fluids (e.g. urine) or exudates (e.g. ascites, cyst fluid, and pleural effusion) contain detached cells that can be cytospun and processed for visualization of structural and morphological changes (Fig. 7). This procedure allows sequential identification of the initial state of cell activation before irreversible cell damage occurs, and further explores a more advanced state of cell injury characterized by nuclear and cytoplasmic abnormalities typical of apoptosis.
The advantage of the proposed procedure is that early morphological changes (membrane blebbing and vesiculation) can be easily identified before anchorage-dependent survival of the cells is compromised[19, 32, 39-42]. In the absence of other morphological or structural modifications, these initial membrane changes indicate a reversible response to stimulation by stressors and can be used as indicators of cell stimulation/suffering. Another advantage is that, from the same culture supernatant or biological fluid, it could be possible to isolate (Fig. 2) and to analyze MPs(Fig. 3A, B).
Finally, *in vivo* proteolytic-dependant degradation of ECM and apoptosis [43-45] may also be studied. In the case of human pathologies or animal experiments this mechanistic procedure has potential application for diagnostic purposes and in the follow up of disease progression and treatment.

In conclusion, we show in this report that plasmin formed at the surface of adherent cells induces two responses(Fig. 7): (1) membrane blebbing and vesiculation (gray arrow)- the early response of adherent cells to proteolytic activation and (2) a delayed response characterized by cell detachment and apoptosis (black arrow). Investigation and analysis of this succession of events identifies the outcome of adherent cells in response to insults that may potentially shift from minimal structural changes (membrane blebbing and vesiculation) to apoptosis. This sequence may be operative in inflammatory processes like atherosclerosis [20] and in adherent cells derived from the central nervous system [16].

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Authorship

LD performed research, analyzed data and wrote the manuscript. LP provided experimental support and participated in manuscript drafting. DGcontributed electron microscopy support.DVdirector of the research unit secured funding. EAC designed the research, analyzed data and wrote the manuscript.

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FIGURE LEGENDS

Figure 1. Identification of plasminogen activators and cellular activation of plasminogen. **A.***Fibrin-agarosezymography* of CHO-K1, HEK-293and HMEC-1cells. The picture was taken after 24 h at 37 °C. The position of purified control references (100 pmol of plasmin: Pn, 1 pmol of tPA and 0.2 pmol of uPA) is indicated on the left. CHO-K1 and HEK-293 areshown to express active tPA whereas HMEC-1 express uPA, identified on the basis of their molecular mobility compared to references (**Upper panel**). Identity of the plasminogen activator was verified by inhibition of their proteolytic activity with specific antibodies (α) on radial diffusion fibrin-agarose gels (**Lower panel**). **B***Kinetics of plasmin formation.*Progress curves of Glu-plasminogen (0.125 and 1µM) activation by CHO-K1 cells.Plasmin formation was detected by measuring the release of *p*-nitroaniline(A_{405}) as a function of time (n = 3). The effect of aprotinin (2 µM) on plasmin activity is shown at 1 µM plasminogen. Background activity in the absence of plasminogen was detected in presence (2 µM) or absence of aprotinin.**C.***Dose-dependent activation of plasminogen.* CHO-K1, HEK-293 or HMEC-1 cells (10^5 cells/well) were incubated with varying concentrations of Glu-plasminogen (0 to 2 µM) and 0.75 mM CBS0065. Kinetics of plasmin formation (ΔA_{405}/min) was followed by measuring the release of *p*-nitroaniline. Data were fitted to the Michaelis–Menten equation. Non-specific activation (k[Pg] in the equation) is proportional to plasminogen concentration. Graph representation of specific activation was obtained by plotting total (raw data) minus non-specific (Km=Vmax/2; Km = 33nM for CHO-K1).The graph represents data for CHO-K1 cells. **D.** The specificity of plasminogen binding and activation was assessed using Glu-plasminogen supplemented, or not, with 50 mMε-ACA, or by pre-treatment of CHO-K1 cells with 50 µg/mLcarboxypeptidase B. Plasmin activity was completely abolished by 0.5 µM aprotinin. Plasmin formation was measured using 0.75 mM CBS0065. Results are expressed in $10^3\Delta A_{405}$/min (mean ± SEM, n = 3).

Figure 2. Early response of cells to plasmin formation: membrane blebbing and vesiculation
CHO-K1,HEK-293 and HMEC-1 cells were incubated with culture medium (CTRL) or with 1 µM plasminogen (Pg) and supernatants were collected after24 h of incubation. Cell response to plasmin was identified by the multiple blebbing of the membrane and vesiculation. **A.** After isolation of these microparticles, their concentration was estimated by measuring the quantity of protein (A_{280nm}). **B.C.D.**The ultra structure of detached cells was observed by electron microscopy. Microparticlesare released before any sign of cell death and have a size of around 300 nm as show at a higher magnification.

Figure 3. Western Blot of microparticles and cell lysate. A. HMEC-1 cells were incubated with 1 µM plasminogen and supernatants were collected after24 h of incubation. Microparticles were isolated as described in the Experimental section.Western blot analysis of uPA (**A**) and plasminogen kringle1 (**B**) were performed with lysates ofmicroparticles. **C.** After incubation with culture medium (CTRL), 1µM plasmin (Pn) or 1µM active-site blocked plasmin (Pn-VFK), cell lysateand culture supernatant were analysed by Western blot using antibodies directed againstlaminin.

Figure 4. Advanced response to plasmin formation: detachment and cell death
Cells cultured in 96-well plate were incubated with 1 µM plasminogen or culture medium for 0 or 72 h. **A.** Optic microscopy after 72h of incubation in the absence (adherent cells, CTRL) or presence (detached, rounded cells) of 1 µM plasminogen. **B.** Quantification of cell death is by MTT assay. After activation, detached cells were pulled-down by centrifugation and MTT

was added. Bars represents the percentage of living cells (%) as compare to control (without plasminogen), n=3±SEM. **C.** DAPI and TUNEL staining of CHO-K1 cells after incubation (72 h) in the presence or absence of 1 µM plasminogen. **D.** Quantification of TUNEL-positive CHO-K1 cells in response to plasminogen exposure for 0 to 72 h. TUNEL index is expressed as the percentage of TUNEL-positive nuclei to DAPI-stained nuclei (bars represent mean ± SEM, n=3).

Figure 5. Advanced response to plasmin formation: electron microscopy of apoptosis. Cells were incubated with plasminogen for 0 to 72 h. **A.**Electron microscopyof control CHO-K1 cells. The normal typical characteristics of cells are detectable: normal nucleus (N), nucleolus (NL) and cytoplasmic appearance including mitochondria (M) with mitochondrial crest, endoplasmic reticulum (RE) and intact membranes. **B.** After incubation for 48 h with 1 µM plasminogen morphological features of early apoptosis were observed: change in nucleus (N) appearance with chromatin condensation into globular or crescent-shaped forms, and formation of lysis vesicles (LV). **C.** After incubation for 72 h with 1 µM plasminogen morphological features of late apoptosis were observed: condensation of mitochondria and high chromatin condensation with compaction and fragmentation of the nucleus.

Figure 6. Plasmin-induced cell detachment upon proteolysis of the extracellular matrix. Cells were incubated with 1 µM plasmin (Pn) or active-site blocked plasmin (Pn-VFK). **A.**After 48 h of incubation the aminolytic activity of plasmin was assessed by addition of CBS0065. Results are expressed in A_{405}/min (mean ± SEM, n = 3).**B.** Quantification of cell death by MTT assay. After activation, detached cells were pulled-down by centrifugation and MTT was added. Bars represent the percentage of living cells (%) as compare to control (without plasmin; mean ± SEM, n = 3)

Figure 7. A sequential procedure to analyze membrane blebbing, vesiculation and cell apoptosis
Near confluent adherent cells were incubated with plasminogen. The native tPAor uPA expressed by these cells allows detection of plasmin formation from 1h of incubation. The plasmin formed at the surface of these cells induces time-dependent responses observed after cytospun and fixation of cells: (1) membrane blebbing and vesiculation,and (2) apoptosis. Culture supernatants were collected, cytospun and submitted to analysis as indicated in methods.

Table 1. From vesiculation to apoptosis
Summary of main data: plasminogen activator, Km, TUNEL index at 48 h (%), viability at 48 h (%) and vesiculation (A_{280}) of control (CTRL) and plasminogen treated (Pg) adherent cells.

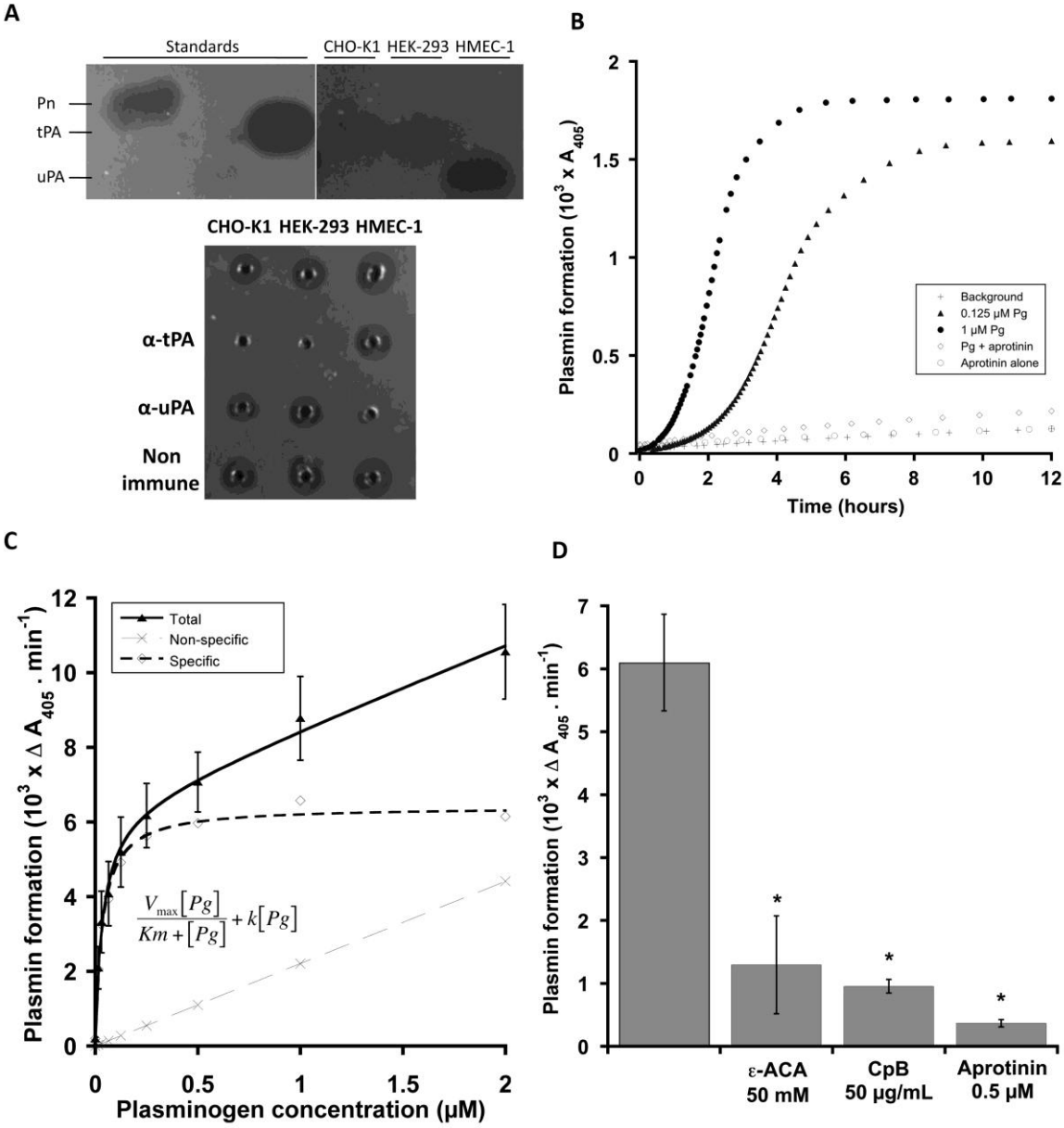
A
Standards
CHO-K1 HEK-293 HMEC-1
Pn
tPA
uPA
CHO-K1 HEK-293 HMEC-1
α-tPA
α-uPA
Non immune
B
Plasmin formation (10³ x A405)
Time (hours)
Background
0.125 μM Pg
1 μM Pg
Pg + aprotinin
Aprotinin alone
C
Plasmin formation (10³ x Δ A405 . min⁻¹)
Plasminogen concentration (μM)
Total
Non-specific
Specific
$\frac{V_{max}[Pg]}{Km+[Pg]}+k[Pg]$
D
Plasmin formation (10³ x Δ A405 . min⁻¹)
ε-ACA 50 mM
CpB 50 μg/mL
Aprotinin 0.5 μM

Figure 1

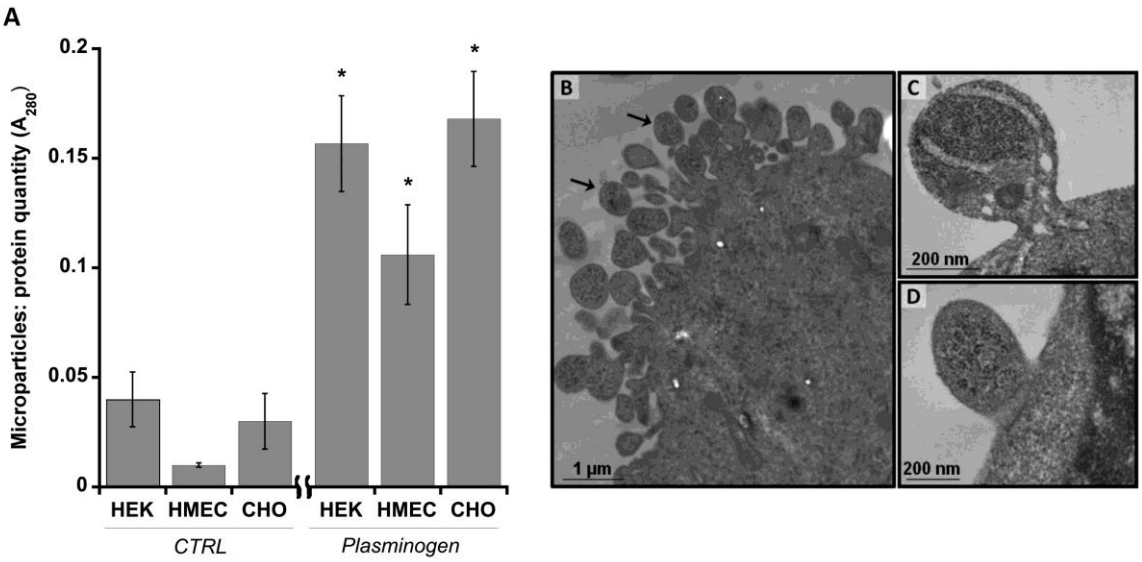
A
Microparticles: protein quantity (A_{280})
0.2
0.15
0.1
0.05
0
*
*
*
HEK
HMEC
CHO
HEK
HMEC
CHO
CTRL
Plasminogen
B
1 µm
C
200 nm
D
200 nm

Figure 2

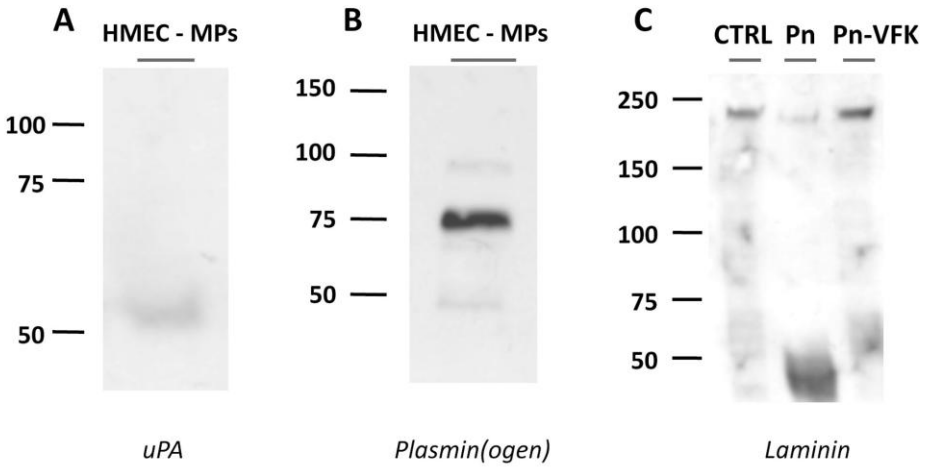
A
HMEC - MPs
100
75
50
uPA
B
HMEC - MPs
150
100
75
50
Plasmin(ogen)
C
CTRL
Pn
Pn-VFK
250
150
100
75
50
Laminin

Figure 3

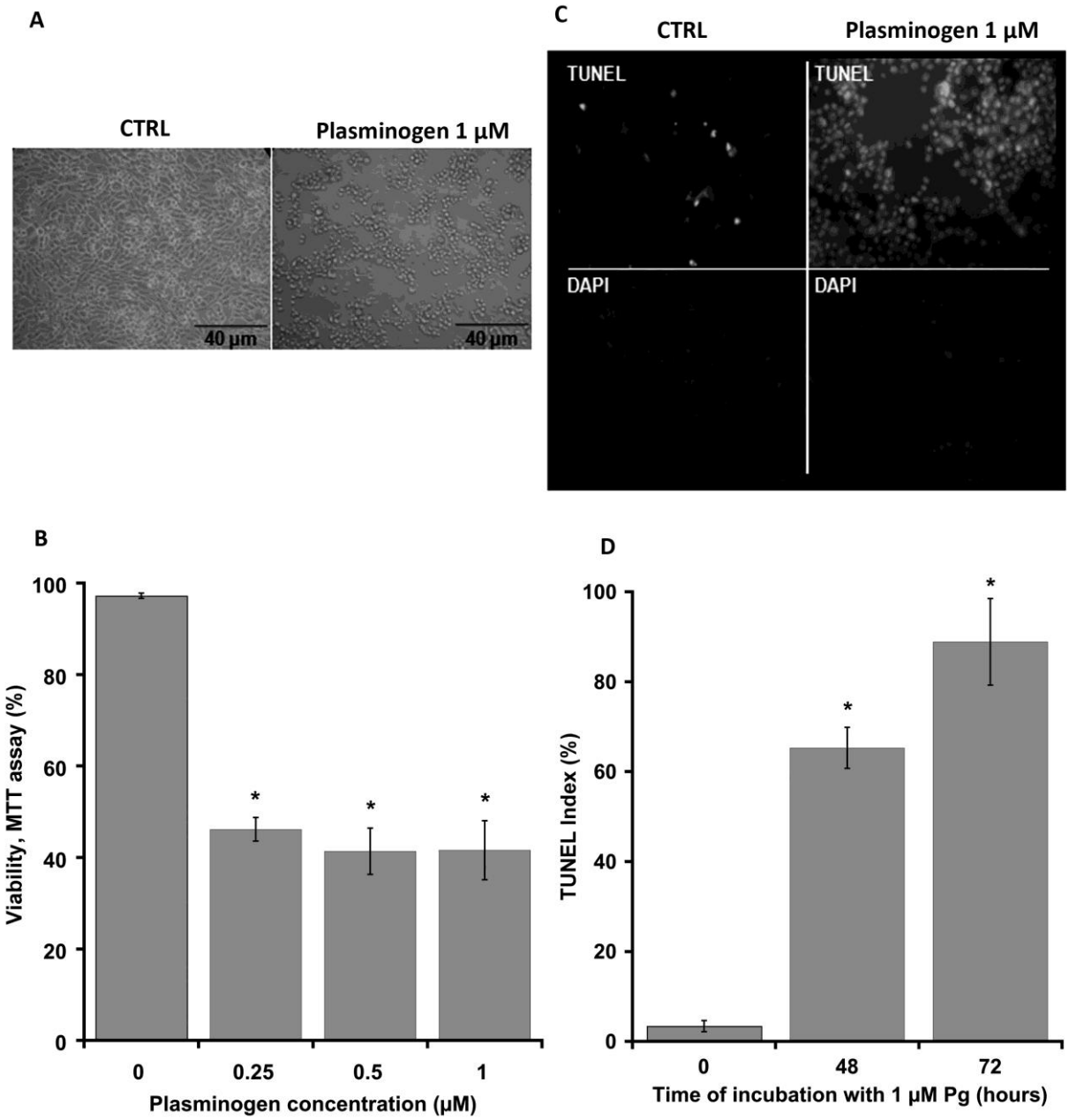
A
CTRL
Plasminogen 1 µM
40 µm
40 µm
C
CTRL
Plasminogen 1 µM
TUNEL
TUNEL
DAPI
DAPI
B
Viability, MTT assay (%)
100
80
60
40
20
0
0
0.25
0.5
1
Plasminogen concentration (µM)
D
TUNEL Index (%)
100
80
60
40
20
0
0
48
72
Time of incubation with 1 µM Pg (hours)

Figure 4

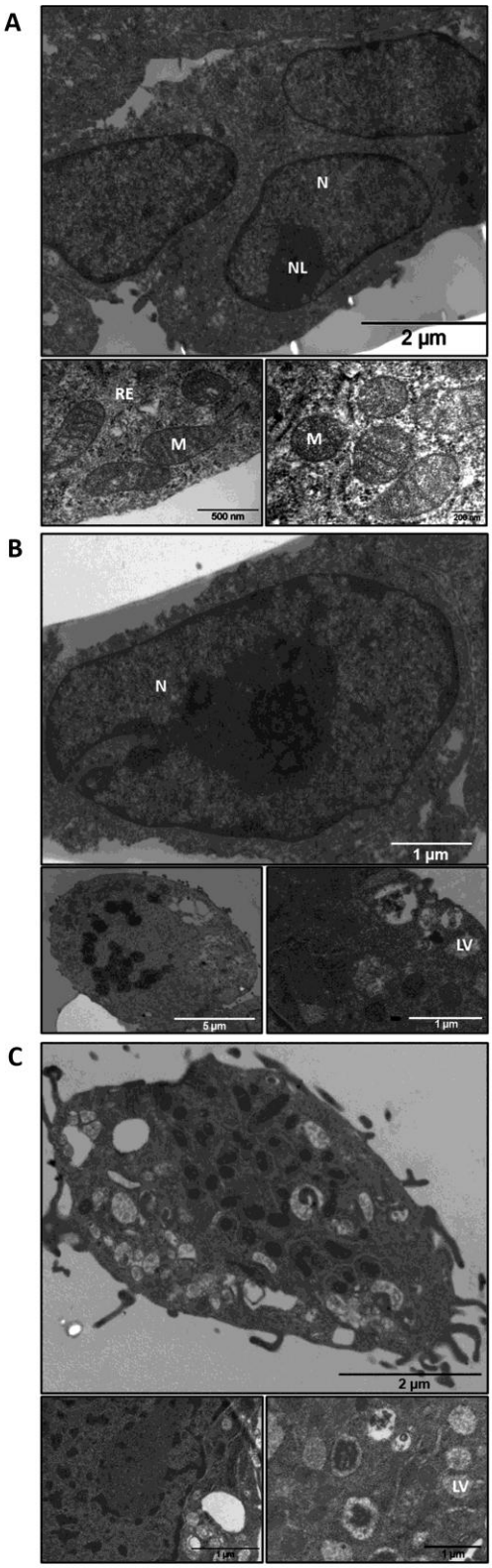

Figure 5

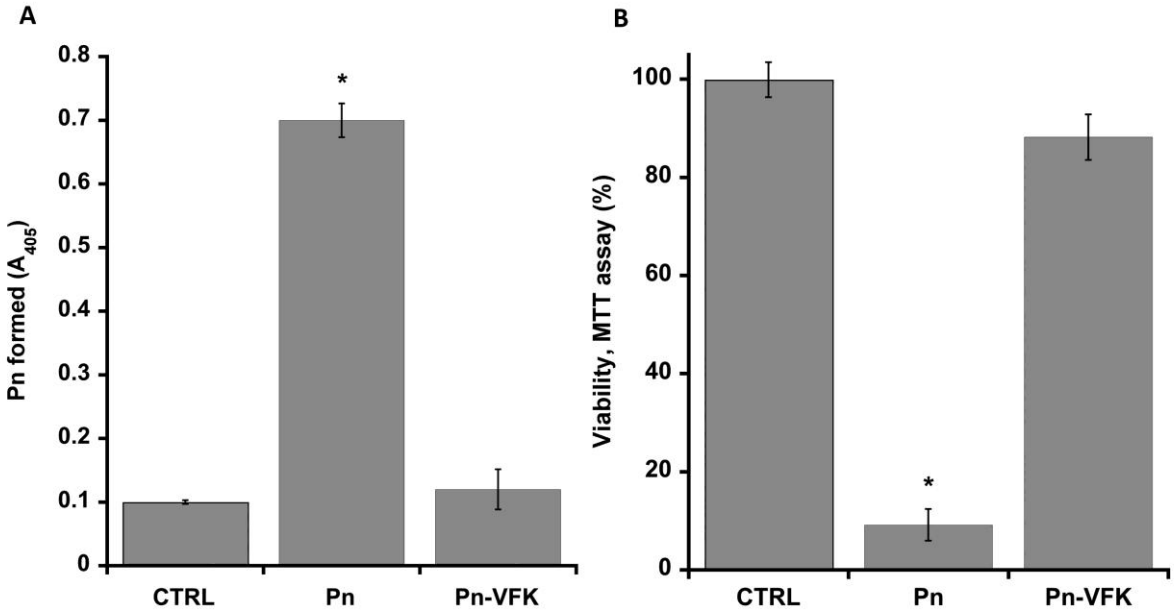
A
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Pn formed (A_{405})
*
CTRL
Pn
Pn-VFK
B
100
80
60
40
20
0
Viability, MTT assay (%)
*
CTRL
Pn
Pn-VFK

Figure 6

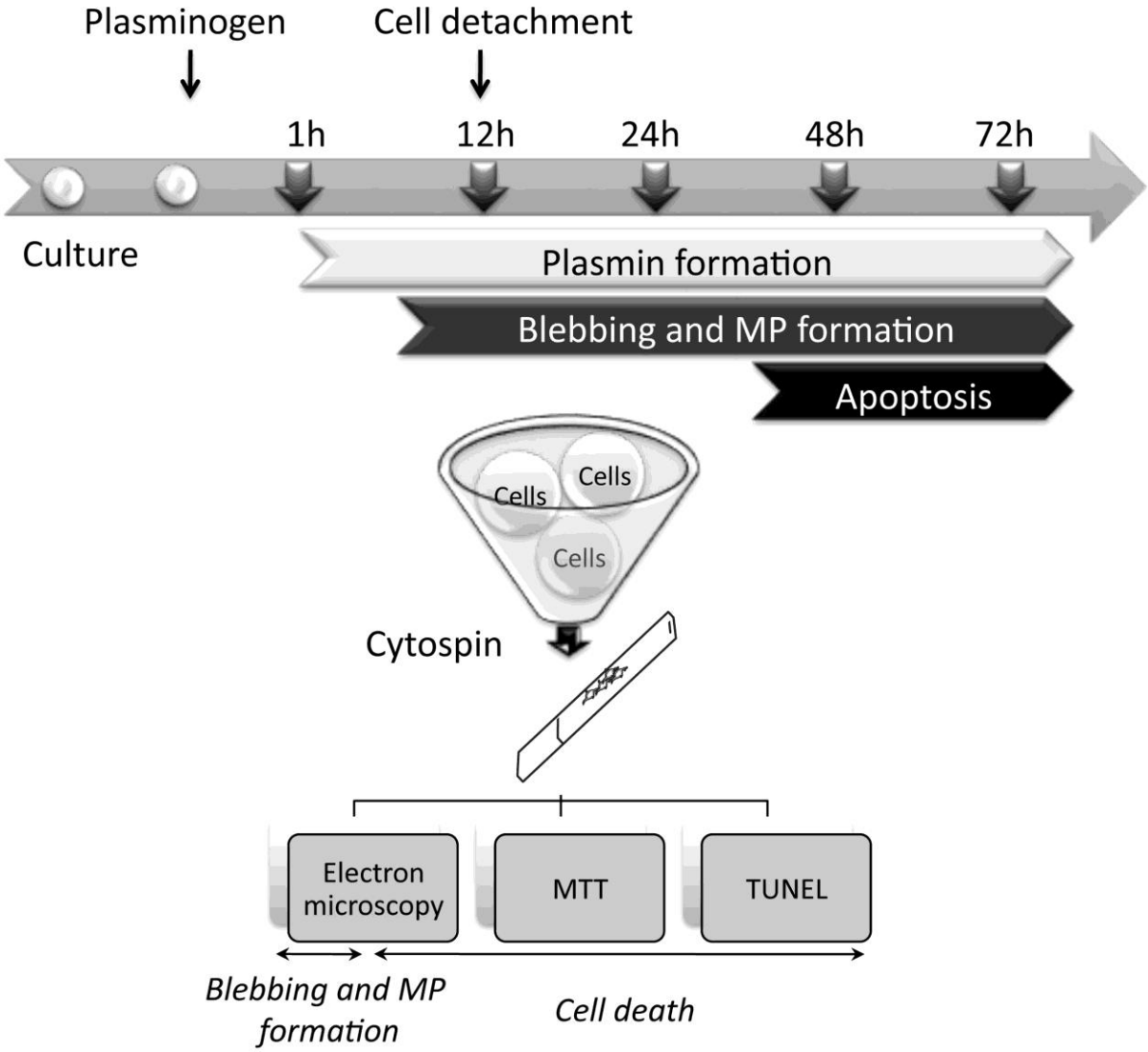
Plasminogen
Cell detachment
1h
12h
24h
48h
72h
Culture
Plasmin formation
Blebbing and MP formation
Apoptosis
Cells
Cells
Cells
Cytospin
Electron microscopy
MTT
TUNEL
Blebbing and MP formation
Cell death

Figure 7

		Activator	Km (nM)	TUNEL index at 48 h (%)	Viabilityat 48 h, MTT assay (%)	Nanovesicles (A 280 nm)
CHO-K1	CTRL	tPA	-	20	99	0.03
	Pg		33	63	42	0.168
HEK-293	CTRL	tPA	-	18	99	0.04
	Pg		31	45	54	0.157
HMEC-1	CTRL	uPA	-	13	99	0.01
	Pg		84	83	66	0.106

Table 1