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Research article

Release of annexin V-binding membrane microparticles from cultured human umbilical vein endothelial cells after treatment with camptothecin

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Abstract

Background: Elevated plasma counts of endothelial microparticles (MP) have been demonstrated in various diseases with a vascular injury component. We used flow cytometry to study the MP-release from cultured human umbilical vein endothelial cells (HUVEC) stimulated by various agonists. MP-release by a topoisomerase I inhibitor camptothecin has been studied in detail.

Results: Overnight stimulation of HUVEC with either LPS or TNFα, or 30 min stimulation with thrombin, phorbol-myristate-acetate, tissue plasminogen activator, or angiotensin-II did not cause a significant release of annexin V-binding MP. In contrast, induction of apoptosis with 5 μM camptothecin, documented by 60–70% desquamation of HUVEC culture, annexin V-binding to the cells and DNA-fragmentation, led to a release of annexin V-binding microparticles (~80,000 MP/10^3 cells). This microparticle-release was prevented by Z-Val-Ala-Asp-fluoromethyl-ketone (ZVAD). Lower concentration of camptothecin (500 nM) induced comparable microparticle-release without loss of the culture confluence and without increase in annexin V-binding to the cells or DNA-fragmentation. Analyzed microparticles were free of nucleic acids and 95% of microparticles were 0.3–1 μm in size. Double-labeling flow cytometry assay showed that all annexin V-binding Microparticles expressed CD59 but only approximately 50% of these also expressed CD105.

Conclusions: Camptothecin treated HUVEC released different populations of annexin V-binding membrane microparticles at early stage after proapoptotic stimulation before detection of phosphatidylserine exposure on the cells or DNA fragmentation. The microparticle-release was ZVAD sensitive but was not enhanced at the executive phase of apoptosis. These observations offer a new insight into microparticle-release as a marker of endothelial stimulation and injury.

Background

Plasma membrane microparticles (MP) of endothelial origin have been identified in normal human blood [1–3] and increased counts of MP carrying endothelial antigens were documented in the blood of patients with lupus anticoagulant [1], acute coronary syndrome [4], thrombotic thrombocytopenic purpura [5] or in patients with exacerbated multiple sclerosis [6]. Similarly to blood platelets, endothelial cells release MP which express phosphatidylserine (PS) on their surface. PS-exposing MP in blood appear to act as the catalytic surface for assembly of the prothrombinase complex [7]. Although circulating MP of

various cellular origin in patient with sepsis were reported to be procoagulant through multiple mechanisms [8,9], in healthy individuals they may have an anticoagulant function by promoting the generation of low amounts of thrombin that activate protein C [2].

The process of membrane vesiculation and MP-release in platelets has been shown to require a calpain-dependent dissociation of membrane proteins from the submembrane cytoskeleton and most likely involves intracellular signaling by platelet protein kinases [10]. Besides platelets stimulated by various agonists such as thrombin with collagen or the complement complex C5b-9 [7,11], a release of PS-containing membrane MP was demonstrated in other cell types such as endotoxin-stimulated monocytes [12], polymorphonuclear leucocytes stimulated with the chemotactic peptide FMLP [13] or apoptotic T-lymphocytes [14]. The normal restriction of PS to the inner surface of the plasma membrane becomes perturbed in apoptotic cells [15,16]. When exposed, PS is a determinant for phagocyte recognition of cells to be cleared. Plasma membrane blebbing and MP-release are generally accepted as typical signs of cell apoptosis [17].

In addition to a possible pathophysiological role of circulating MP, analysis of MP derived from endothelial cells may be a valuable diagnostic marker of vessel wall injury. The phospholipid-binding protein annexin V has a high affinity for PS [18] and is utilized in MP detection in blood and in cell supernatants [1]. Since very little is known about the mechanism of MP-release from endothelial cell, it is difficult to judge the type and extent of endothelial injury represented by increased plasma counts of endothelial MP. We used flow cytometry to study the release of annexin V-binding membrane microparticles from cultured human umbilical vein endothelial cell (HUVEC) stimulated by various agonists. We further focused our study on MP-release from HUVEC after proapoptotic stimulation with the topoisomerase I inhibitor camptothecin (CPT). Since CPT analogues become one of the most promising new classes of antineoplastic agents introduced into the clinic in recent years [19], activity of camptothecin on endothelial cells may be of great interest.

Results

With the exception of high concentration of ionophore A23187, rapid stimulation of HUVEC with various agonists did not result in a release of annexin V-binding microparticles

Flow cytometry analysis of HUVEC culture supernatant demonstrated that a 30 min treatment with 10 μM ionophore A23187 caused the release of low counts of annexin V-binding MP (Fig. 1A). Similar short-time activation of HUVEC with phorbol myristate acetate (PMA) (1 μg/mL), human thrombin receptor activating peptide (TRAP) 1–50 μmol/L, human thrombin (THR) 0.1–10 NIH Unit/mL, human tissue plasminogen activator (TPA) 3.5 kU/mL or human angiotensin II (AGTII) 1 μmol/L did not cause any significant increase in MP-release (Fig. 1A). All of the agonists applied induced morphological changes in HUVEC seen by phase contrast light microscopy, which confirmed the cellular activation (not shown).

Proapoptotic, but not proinflammatory, stimulation induced a release of annexin V-binding microparticles from HUVEC

MP count increased overnight in the media of unstimulated cell culture, but this was not enhanced by TNFα (1000 U/ mL) or by LPS (10 μg/mL). However, when cells were activated with TNFα in the presence of cycloheximide (50 μg/mL) to promote apoptosis [20], a dramatic increase in MP count was observed, as compared to TNFα or cycloheximide treatment alone (Fig. 1B). Similarly, induction of apoptosis with 5 μM CPT, a topoisomerase I inhibitor [19], led to a release of annexin V-binding MP which was prevented by ZVAD. ZVAD also inhibited a release of MP from unstimulated cells, which probably represents spontaneous apoptosis of cultured HUVEC (Fig. 1B).

MP-release did not correlate with the level of cell apoptosis in HUVEC stimulated with camptothecin

Phase contrast microscopy showed that HUVEC culture treated for 24 hrs with 5 μM CPT (Fig. 2C) lost confluence with about 30–40% cells remaining adherent. The level of apoptosis of adherent cells was assessed by using TUNEL and annexin V-FITC/ propidium iodide assays (Fig 3). Approximately one third of the remaining adherent cells showed annexin V-binding (Fig. 3B) and DNA-fragmentation (TUNEL-positivity) (Fig. 3C). Interestingly, a ten times lower concentration of CPT (500 nM) also induced significant MP-release after only 4 hrs of treatment (Fig. 3A) and after 24 hrs, 500 nM camptothecin induced MP-release comparable to 5 μM CPT treatment, but without any significant increase in annexin V-binding to the cells or DNA-fragmentation (Fig. 3B,3C). The cell culture was still confluent (Fig. 2B) with about 20–30% lower cell density as compared with 0.5% DMSO treated HUVEC (Fig. 2A). This result indicates that MP-release is not related to cell desquamation. To investigate whether maturation of the cell culture influences the degree of MP-release from HUVEC in response to CPT, we compared MP-release from subconfluent HUVEC (50% of confluence) at 24 hrs in culture, HUVEC reaching confluence at 48 hrs in culture and overgrown HUVEC at 72 hrs in culture. Our results showed that MP-release in response to CPT declined with culture maturation. There was also a change of the sensitivity of overgrown HUVEC to CPT treatment in confluent 72 hrs old culture resulting in the higher con-

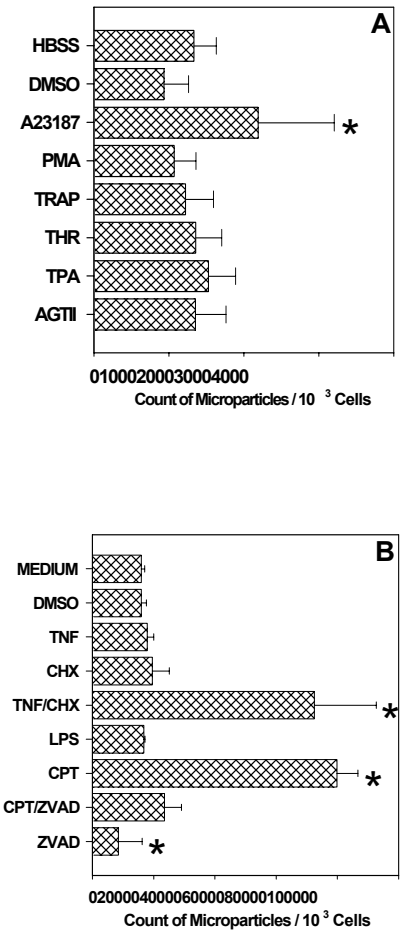

Figure 1

Release of annexin V-binding microparticles from HUVEC after stimulation with different agonists **A) Short-time stimulation (30 min):** confluent HUVEC at 48 hrs in culture were incubated with buffer HBSS/BSA only (HBSS), 0.5 % DMSO in HBSS/BSA (DMSO), 10 μM A23187 (A23187), PMA 1 μg/mL (PMA), 10 μM thrombin receptor activating peptide (TRAP), thrombin 1 NIH U/mL (THR), tPA 3.5 kU/mL (TPA), or with 1 μM angiotensin II (AGTII). **B) Overnight stimulation (24 hrs):** confluent HUVEC at 48 hrs in culture were incubated with medium only (MEDIUM), 0.5 % DMSO in medium (DMSO), TNFα 1000 U/mL (TNF), cycloheximide 50 μg/mL (CHX), TNFα 1000 U/mL with cycloheximide 50 μg/mL (TNF/CHX), lipopolysaccharide E. coli 055:B5 10 μg/mL (LPS), 5 μM camptothecin (CPT), 5 μM camptothecin with 50 μM Z-VAD-fluoromethyl ketone (CPT/ZVAD), or with 50 μM Z-VAD-fluoromethyl ketone. After incubation, supernatant from cell culture was harvested and annexin V-FITC-positive MP were counted. Means of 4 independent experiments ± SD are presented, * = $p < 0.05$ vs. DMSO (ANOVA).

centration of CPT (5 μM) releasing higher levels of MP (Fig. 4). In growing culture at 24 hrs, 500 nM CPT caused even higher MP-release than 5 μM CPT. In confluent HUVEC 48 hrs old, the MP-release induced by 500 nM and 5 μM CPT was not significantly different (Fig. 4).

Endothelial antigens CD 105 and CD 59 were both expressed on living cells, apoptotic bodies and other cell fragments in CPT treated HUVEC culture but CD 105 was absent on 50% of annexin V-binding membrane microparticles

Using flow cytometry, in SSC vs. FSC plot we gated homogeneous population of single living cells (G1 on Fig. 5A), which appeared negative in propidium iodide staining (Fig. 5B). Living cells from control cultures as well as from cultures treated with CPT were strongly positive for both antigens CD 105 and CD 59 (Fig. 5C). Expression of CD59 on endothelial cells was not influenced by 24 hrs incubation with 500nM or 5μM CPT as compared with control cells incubated with 0.5% DMSO. Expression of CD105 was only slightly decreased on CPT treated cells. Cell fragments > 4 μm in size as compared with standard beads were gated separately (G2 on Fig. 5A). Larger cell fragments appeared positive in propidium iodide staining (Fig. 5B). All analyzed cell fragments were positive for CD59 and also expressed CD 105 (Fig. 5D). Comparison of areas under the different peaks on the histogram (Fig. 5C,5D) indicates that the portion of cell fragments corresponding to apoptotic bodies and necrotic cells was significantly higher in culture treated with 5 μM CPT as compared to culture treated with 500 nM CPT or with 0.5% DMSO alone. In a separate protocol we analyzed the expression of CD 59 and CD 105 on membrane MP. Comparison of FSC values of standard beads and analyzed MP showed that 95% of annexin V-binding MP released from HUVEC were < 1 μm in size (range 0.3 – 3.0 μm). Analyzed MP were free of nucleic acids as they were not stained with propidium iodide (not shown). Double-labeling flow cytometry assay showed that all annexin V-binding membrane MP expressed CD59 but only approximately 50% of these expressed CD 105 (Fig. 6).

Discussion

Our previous study demonstrated that cultured HUVEC released annexin V-binding membrane MP in response to proapoptotic stimulation with CPT [3]. In the present study, we investigated whether endothelial cells release membrane MP after rapid stimulation with Ca^{2+}-mobilizing agents or after overnight stimulation with proinflammatory agents TNFα and LPS, or only after induction of apoptosis. Our negative results with thrombin, TRAP, PMA, TPA and AGTII indicate that rapid Ca^{2+}-mobilization, despite dramatic morphological changes of the cells, does not result in MP-release from HUVEC (Fig. 1A). The only report showing MP-release after rapid stimulation

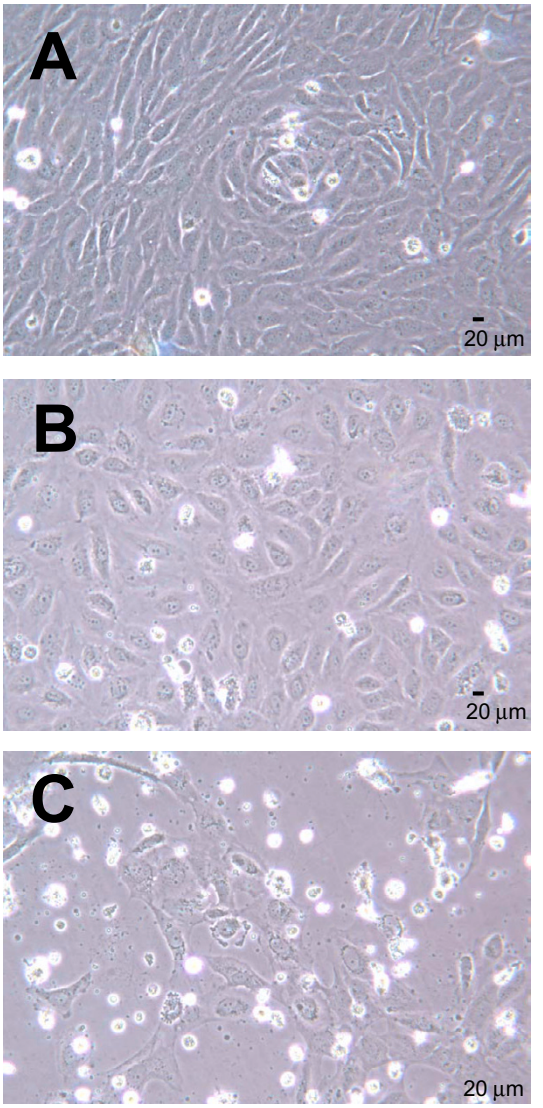

Figure 2
Phase contrast photomicrographs of HUVEC culture after treatment with camptothecin Confluent HUVEC at 48 hrs in culture were treated for 24 hrs with (A) 0.5 % DMSO: confluent culture, (B) 500 nM CPT: confluent culture with about 20–30% lower cell density as compared with A ; (C) 5 µM CPT : apoptotic culture with about 60–70 % desquamation, bright objects represent floating cell fragments including apoptotic bodies. Phase contrast photomicrographs (200 ×) are shown.

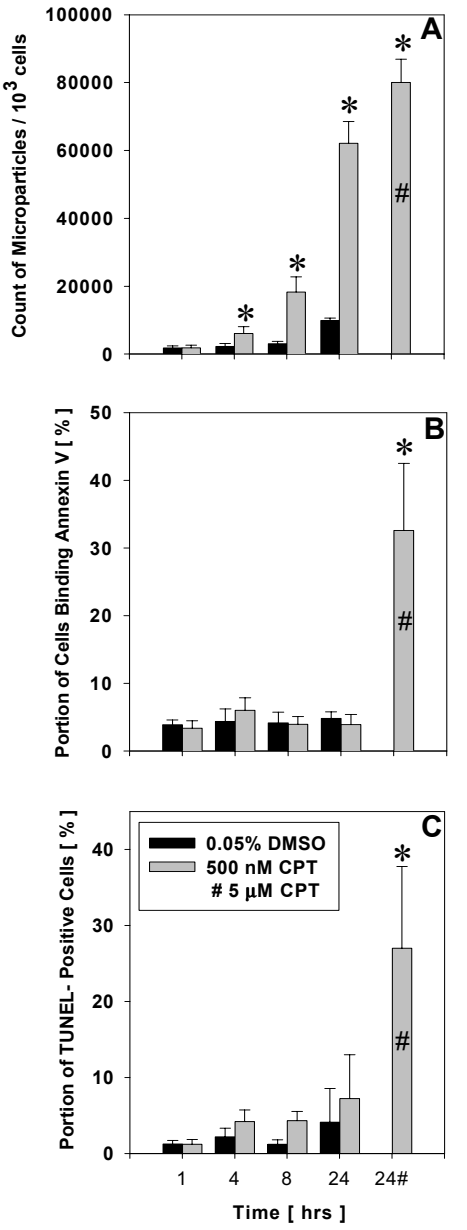

Figure 3
Release of annexin V-binding membrane microparticles and the level of cell apoptosis in HUVEC stimulated with camptothecin. Confluent HUVEC at 48 hrs in culture were incubated with 0.5 % DMSO or with 500 nM or 5 µM CPT in medium. After indicated time periods supernatant from cell culture was harvested and annexin V-FITC-positive MP were counted (A). Adherent cells were harvested and portions of cells binding annexin V (propidium iodide negative) (B) and portions of TUNEL-positive cells (C) were evaluated. Means of 4 experiments ± SD are presented, * = $p < 0.05$ vs. DMSO (ANOVA).

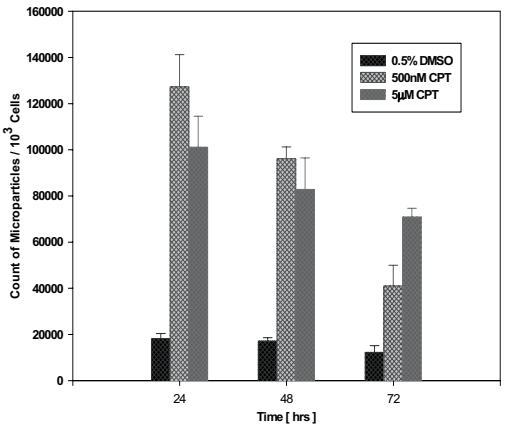

Figure 4
Release of annexin V-binding membrane microparticles in subconfluent, confluent and overgrown HUVEC culture treated with camptothecin HUVEC at 24 hrs (50% of confluence), 48 hrs (reaching confluence) or 72 hrs (overgrown) in culture were incubated for 24 hrs with 0.5 % DMSO, 500 nM or 5 μM CPT in medium. After incubation supernatant from cell culture was harvested and annexin V-FITC-binding MP were counted. Means of 4 experiments ± SD are presented, significant differences ($p < 0.05$, ANOVA): 500 nM CPT – 24 hrs vs. 48 hrs, 48 hrs vs. 72 hrs, 24 hrs vs. 72 hrs; 5 μM CPT – 24 hrs vs. 48 hrs, 24 hrs vs. 72 hrs; 24 hrs – 500 nM CPT vs. 5 μM CPT; 72 hrs – 500 nM CPT vs. 5 μM CPT.

with a physiologic agonist is the study by Hamilton *et al.* [21] that demonstrated MP-release from HUVEC with the complement complex C5b-9. This membrane-attack complex may destabilize cell membrane by generating pores in phospholipid bilayer. We observed MP-release from HUVEC after short-time treatment only with high concentrations (10 μM) of A23187. In this experiment, a direct destabilizing effect of the Ca^{2+}-ionophore A23187 on cell membrane resulting in MP formation is also likely. Another question is relation of plasma membrane blebbing and MP-release. One study documented peroxide-induced membrane blebbing after a short time stimulation of endothelial cells [22]. Although MP-release was not investigated, authors showed micrographs of stimulated cells with membrane blebs of about 3 μm in diameter. Size of majority of MP produced in our experiments is less than 1μm. This difference indicates that the mechanism of MP-release may be different from membrane blebbing visually observed on cells by conventional light microscopy.

We did not find any significant MP-release from HUVEC after overnight stimulation with TNFα (Fig. 1B) although stimulation of the HUVEC culture was confirmed by up-regulation of CD 54 on cell surface (not shown). In comparison, Combes *et al.* [1] recorded a four fold increase in MP count in HUVEC culture stimulated overnight with TNFα when compared to control cells. These discrepant results may stem from differences in HUVEC culture conditions and the flow cytometric assay. In our study, the proapoptotic stimulation was the only efficient way to release membrane MP from HUVEC. Since MP-release from endothelial cells after proapoptotic stimulation was not studied before, we focused on its characterization. We used CPT, an inhibitor of topoisomerase I, that is a well defined apoptogenic agent belonging to a promising class of antineoplastic drugs [23]. In contrast to more physiological stimuli (i.e, oxidative stress) induction of apoptosis by CPT is less complex and thus is a better model for study of MP-release phenomenon. Our results showed that the growth stage of HUVEC culture influenced MP release in response to CPT (Fig. 4). It is difficult to understand why 500 nM CPT caused higher MP-release than 5 μM CPT in subconfluent HUVEC. This difference is small and may be influenced by error of analysis. It is evident, however, that cell culture maturation increased resistance to camptothecin treatment. This may be related to decreased DNA synthesis in mature confluent HUVEC.

Size distribution of annexin V-binding MP released from HUVEC after proapoptotic stimulation was similar to that of endothelial MP found in blood [1]. The small size and the absence of nucleic acids indicate that the analyzed MP are different from apoptotic bodies formed in the terminal stage of apoptosis. Our finding of CD59+ CD105+ and CD59+CD105- populations of MP (Fig. 6) suggests that HUVEC can release different types of annexin V-binding MP with respect to their antigen expression. This observation cautions against the use of a single antigen for detection of endothelial MP in blood. The presence of CD59 on all annexin V-binding MP corresponds to the high expression of this glycosylphosphatidylinositol anchored glycoprotein on endothelial cells. The homodimeric transmembrane glycoprotein CD 105 (endoglin) is relatively specific for endothelial cells and is abundant with more than 500,000 copies per cell on HUVEC [24]. It is not clear, however, why they are CD59-positive MP that express CD105 and some that do not (Fig. 6). Although CD 105 was shown to be an endothelial cell proliferation marker [25], its expression was only slightly decreased on CPT treated cells (Fig. 5C) and was stable on overgrown confluent HUVEC up to 96 hrs in culture (not shown). We also found this antigen on cell fragments, including apoptotic bodies (Fig. 5D). Since CD59 is a protein with an important complement regulatory function, and CD105 is a component of the receptor for TGFβ, there may be functional and/or diagnostic differences in the MP populations.

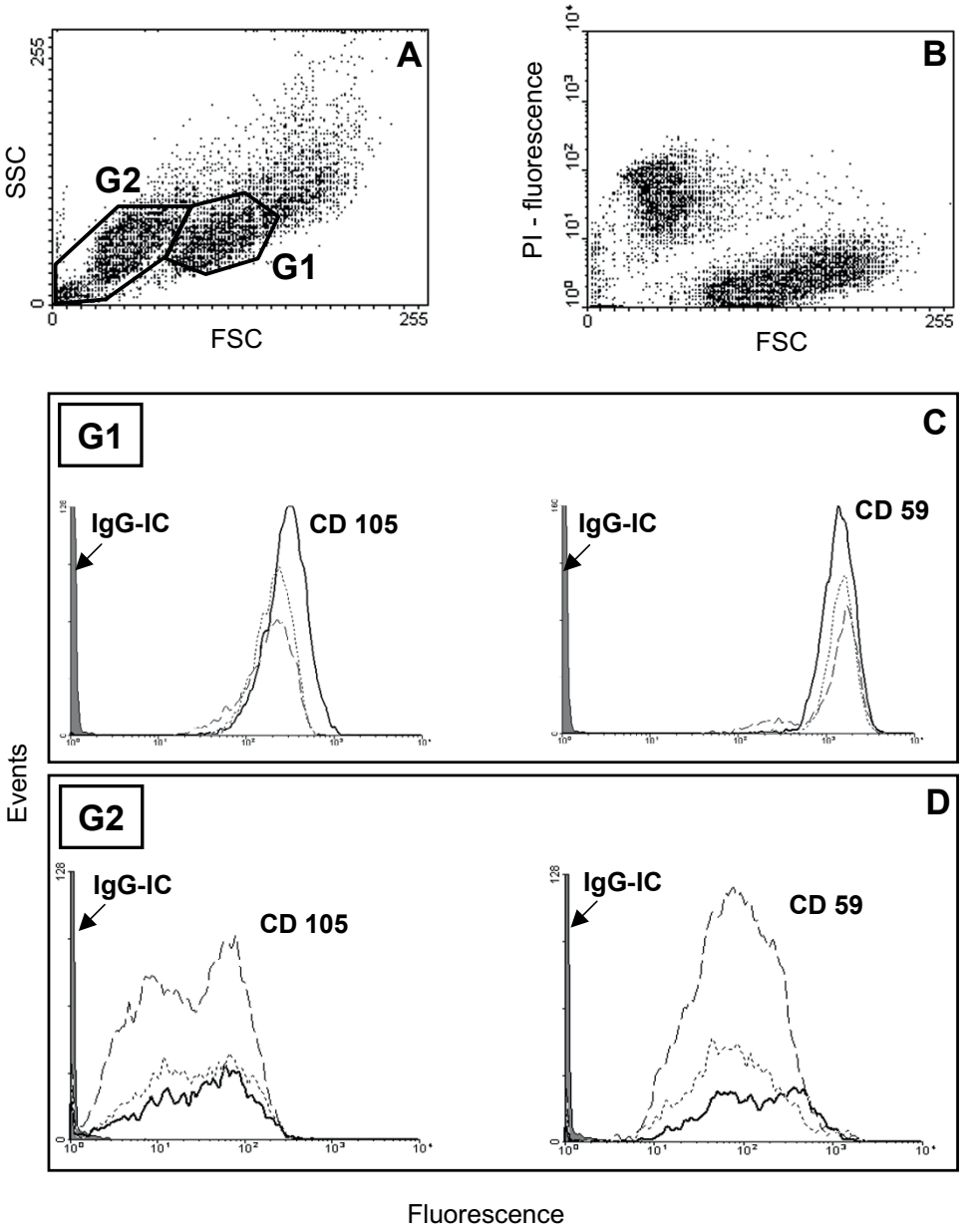

Figure 5

Expression of CD59 and CD105 on endothelial cells and apoptotic bodies in HUVEC culture treated with camptothecin Confluent HUVEC at 48 hrs in culture were treated for 24 hrs with 0.5% DMSO, 500 nM or 5 μM CPT in medium. After treatment the cell culture supernatant was pooled with harvested adherent cells. Mixture of cells, apoptotic bodies and other cell fragments was labeled with propidium iodide (PI) or with PE-labeled Mabs to CD59 or CD105 and analyzed by flow cytometry. Forward scatter (FSC) vs. side scatter (SSC) plot (A) shows gated intact single cells (G1) and apoptotic bodies and other cell fragments (G2) in a sample treated with 0.5% DMSO alone. PI – fluorescence vs. FSC plots of this sample is shown (B). Histograms showing expression of CD105 and CD59 on cells (C) and cell fragments (D) from HUVEC treated with 0.5% DMSO (solid line), 500 nM CPT (dotted line) or 5 μM CPT (dashed line) are shown. Binding of the IgG2a isotype control (IgG-IC) corresponded to the fluorescence of nonlabeled cells and cell fragments. Results are representative of three experiments.

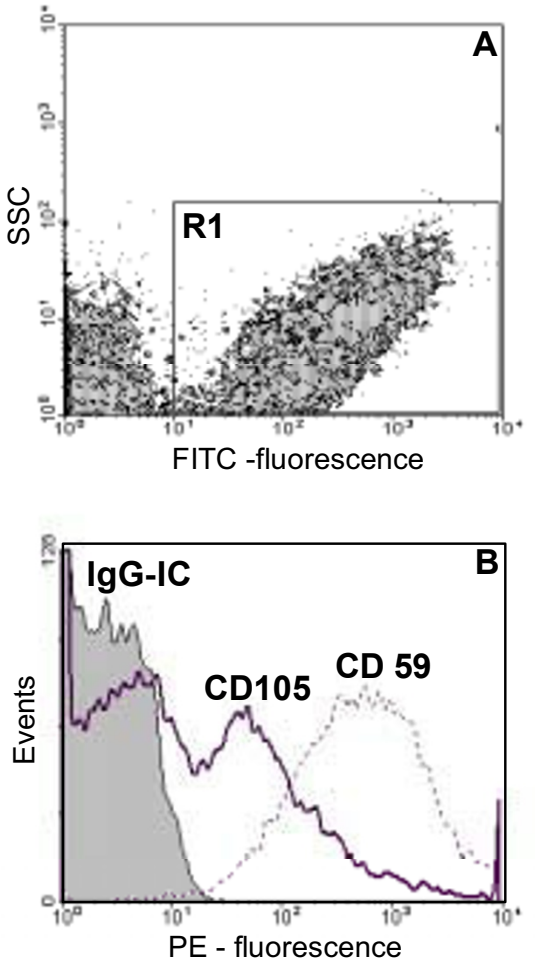

Figure 6
Expression of CD59 and CD105 on annexin V-binding HUVEC microparticles. HUVEC at 48 hrs in culture were incubated for 24 hrs with 5 μM CPT. After incubation MP in culture medium were harvested and double-labeled with annexin V-FITC and a saturating concentration of a PE-conjugated Mab : IgG2a isotype control (IgG-IC, filled area), binding of the isotype control corresponded to the fluorescence of nonlabeled MP; Mab to CD59 (dashed line); Mab to CD105 (thick line). After incubation and washing, samples were analyzed by flow cytometry. The annexin V-FITC positive MP were gated in R1 gate in FITC-flourescence vs. SSC plot (A) and their binding of PE-labeled Mabs was evaluated in PE-fluorescence histogram plot (B). Results are representative of six experiments.

It is generally accepted that appearance of PS on the outside of the plasma membrane is an early sign of apoptosis in many cell types after different treatments [26]. Chan *et al*[27] showed that exposure of PS on the cell surface preceded DNA strand breaks in apoptosis of different cell lines. Cell membrane vesiculation and release of MP is considered to belong to the execution stage of apoptosis where the fate of the cell is sealed. To our surprise, in CPT treated HUVEC the MP-release did not correlate with the extent cell desquamation and apoptosis or remaining adherent cells characterized by PS exposure on the cell membrane and DNA-fragmentation (TUNEL) (Fig. 3). In contrast, Aupeix *et al*. [17] reported that the release of PS-containing MP was proportional to hypodiploid DNA in THP-1 and U-937 cells stimulated by various apoptotic agents. Our experiments with 500 nM CPT treated HUVEC indicate that annexin V-binding MP may be released from adherent cells at initial phase of apoptosis before exposure of PS on cell surface or DNA fragmentation. Cell desquamation and massive formation of apoptotic bodies seen after 5 μM CPT treatment did not further enhance the MP-release (Fig. 3) thus supporting the hypothesis that annexin V-binding MP-release takes place in the initial phase of apoptosis. The essential step in plasma membrane destabilization and MP-release very likely is a caspase-dependent sphingomyelin hydrolysis, which leads to the production of ceramide [28–30]. It is possible, however, that sphingomyelin hydrolysis and MP-release may be a consequence of the action of initiator caspases or other ZVAD sensitive proteases rather than only executioner caspases [15,31,32]. Our study was performed in HUVEC culture, which may have particular properties in respect of MP release when compared with endothelial cells from other organs. Our finding that the PS is not exposed on the cells treated with low concentration of CPT while they release PS-containing MP may suggests that these cells release MP to remove PS from the cell surface as a protective mechanism that may counteract proapoptotic stimulation in the life/death decision process.

Conclusions

Our results demonstrate that HUVEC release different populations of annexin V-binding membrane MP in response to proapoptotic stimulation. We showed that rapid stimulation of HUVEC with thrombin, PMA, TPA or AGT II or overnight stimulation with TNFα or LPS does not induce release of annexin V-binding membrane MP. Release of MP after treatment with camptothecin very likely occurs at early stage prior to a "point of no return" in apoptosis. This phenomenon may represent a physiologic repair process rather then be a part of apoptotic cell death.

Materials and methods

Monoclonal antibodies

Phycoerythrin (PE) conjugated IgG2a isotype control (IgG-IC) was obtained from Immunotech, (Marseille, France). Mab to human CD59 (PE-conjugated, clone MEM 43) was from Caltag Lab. (Burlingame, CA), Mab to

human CD105 (PE-conjugated, clone N1-3A1) was from Ancell/Alexis (San Diego, CA).

Endothelial cell culture

HUVEC were obtained from Clonetics (San Diego, CA) and cultured in endothelial cell growth media EGM-2 (Clonetics) containing 2% of fetal bovine serum and supplements. Cells of 2nd passage were used in the experiments. Cells were seeded at a density of 10,000 cells/cm^2 in polystyrene Falcon six-well plates or 60 mm dishes (Becton Dickinson Labware, Franklin Lakes, NJ) and maintained at 37°C under an atmosphere of 5% of CO_2 and 95% room air. Cells were used for experiments at 48 hrs in culture when they reached confluence. For apoptotic assays, treated culture was washed with Hanks' balanced salt solution (HBSS) with 0.35% bovine serum albumin (HBSS/BSA) and adherent cells were harvested with 20 mM HEPES buffer with 100 mM NaCl, 0.5% BSA and 10 mM EDTA, pH 7.4. After harvest, cells were centrifuged (300 g/5 min), washed with HBSS/BSA and used for analysis.

Phase contrast microscopy

HUVEC cultures in 6-well plates were photographed using CK40 Olympus culture microscope (Olympus, Japan) with a SPOT camera and software (Diagnostic Instruments, Inc., MI, USA). Phase contrast at 200× magnification was used. The culture cell density was estimated by visual counting of cells in a standard area on the micrographs.

Overnight stimulation of HUVEC

Confluent HUVEC were washed with HBSS and incubated for 24 hrs at 37°C with various agonists in the medium: MEDIUM only; DMSO 0.5 % (vehicle for camptothecin); tumor necrosis factor-α (TNF) from Alexis Biochemicals (San Diego, CA) 100 or 1000 U/mL; cycloheximide (CHX) 50 μg/mL; TNF 1000 U/mL with cycloheximide 50 μg/mL (TNF/CHX); lipopolysaccharide (LPS) *E. coli* 055:B5 from Calbiochem (San Diego, CA) 10 μg/mL; camptothecin (CPT) from Sigma (St. Louis, MO) 5 μmol/L or 500 nmol/L; CPT with caspase inhibitor Z-Val-Ala-Asp-fluoromethyl ketone (ZVAD) from Calbiochem (San Diego, CA) 50 μmol/L or ZVAD 50 μmol/L alone. After incubation, media and cells were harvested for analysis.

In a study of MP-release in subconfluent, confluent and overgrown HUVEC culture, the cells at 24 hrs (50% of confluence), 48 hrs (reaching confluence) or 72 hrs (overgrown) in culture were incubated for 24 hrs with 0.5 % DMSO, 500 nM or 5 μM CPT in medium. After incubation supernatant from cell culture was harvested and annexin V-FITC-binding MP were counted.

In a study of expression of CD59 and CD105 on endothelial cells, apoptotic bodies and other cell fragments, the confluent HUVEC at 48 hrs in culture were treated for 24 hrs with 0.5% DMSO, 500 nM or 5 μM CPT in medium. After treatment the cell culture supernatant was separated, adherent cells were harvested with 20 mM HEPES buffer with 100 mM NaCl, 0.5% BSA and 10 mM EDTA, pH 7.4, and pooled with culture supernatant. This mixture of cells, apoptotic bodies and other cell fragments was centrifuged (300 g/5 min), washed with HBSS/BSA, labeled in aliquots with saturating concentration of PE-conjugated Mab against CD105 or CD59, washed again in HBSS/BSA and analyzed by flow cytometry.

Short-time stimulation of HUVEC

Confluent HUVEC were washed with HBSS and incubated for 30 min at 37°C with various agonists in HBSS with 0.35%BSA: HBSS alone; 0.5 % dimethyl sulfoxide (DMSO); ionophore A23187 1–100 μmol/L; phorbol myristate acetate (PMA) 1 μg/mL; human thrombin receptor activating peptide SFLLRNP (TRAP) 1–50 μmol/L; human thrombin (THR) US FDA standard lot J (Bethesda, MD) 0.1–10 NIH Unit/mL; human tissue plasminogen activator (TPA) 3.5 kU/mL or human angiotensin II (AGTII) 1 μmol/L. All chemicals were from Calbiochem (San Diego, CA) unless otherwise stated. After incubation, cell cultures were observed by phase contrast microscopy and cell supernatants were collected for MP analysis.

Annexin V-labeling of microparticles

One hundred μL of medium or cell culture supernatant were incubated with 4 μL of annexin V-FITC from BD PharMingen (San Diego, CA) for 20 min at room temperature with or without 50 mM EDTA to estimate the non-specific binding. Fifty μL of labeled MP were added to Tru-Count tubes (Becton Dickinson) containing standard beads in 450 μL of HBSS/BSA solution. Samples were immediately analyzed with flow cytometry.

Immunolabeling of annexin V-binding microparticles

Medium or cell supernatant containing MP were centrifuged at 2,700 g for 5 min, and the sediment was discarded. Supernatant was further centrifuged at 19,800 g for 5 min at 10°C, and sedimented MP were resuspended in the original volume of HBSS/BSA. Aliquots of 50 μL were incubated for 20 min at room temperature with 2 μL of annexin V-FITC from BD Pharmingen (San Diego, CA) and saturating concentrations of PE-conjugated Mab. After incubation and washing with 1 mL HBSS/BSA (centrifuged at 19,800 g for 5 min at 10°C) samples were diluted with 450 μL HBSS/BSA and analyzed by flow cytometry.

Flow cytometry of endothelial cells and cell fragments

Cell samples were analyzed by a FACScan flow cytometer (Becton Dickinson, San Diego, CA) equipped with CEL-

LQUEST software with forward scatter (FSC) and side scatter (SSC) in linear mode setting. Samples were analyzed using forward scatter (FSC) vs. side scatter (SSC) plot and propidium iodide (PI) fluorescence vs. FSC plot. Single cells were gated separately in gate G1 and apoptotic bodies and other cell fragment in gate G2 (Fig. 5). Count of 10,000 gated particles was analyzed for each sample. Apoptotic status of cells was assayed using Annexin V-FITC Apoptosis Detection Kit for annexin V/ propidium iodide cell labeling and using Apo-Direct Kit for terminal deoxynucleotidyltransferase dUTP-FITC nick end labeling (TUNEL) according to manufacturer's instruction (BD Pharmingen, San Diego, CA). Percentages of propidium iodide-negative cells binding annexin V and TUNEL-positive cells were evaluated. In a study of the expression of CD59 and CD105 on HUVEC, histograms showing expression of CD105 and CD59 on cells (G1) and cell fragments (G2) are presented. Binding of the IgG2a isotype control (IgG-IC) corresponded to the fluorescence of non-labeled cells and cell fragments. Standard Quantum TM24 beads (Flow Cytometry Standards, San Juan, PR) were run each day as a separate sample to standardize fluorescence readings.

Flow cytometry analysis of endothelial cell membrane microparticles

MP were analyzed in a separate protocol [3] using a modified method of Combes et al. [1]. Annexin V-FITC-positive MP were gated in a SSC vs. fluorescence logarithmic plot. For MP quantitation, TruCount beads from Becton Dickinson (San Diego, CA) were used in each sample as an internal standard. TruCount beads were gated in a SSC vs. FSC logarithmic plot and analysis was stopped when 5,000 beads were counted. MP count released by 10^3 cells was calculated for each sample. TetraSpeck fluorescent microsphere standards of 0.2, 0.5, 1.0 and 4.0 μm in diameter from Molecular Probes (Eugene, OR) were used to estimate MP size based on comparison of FSC values. Antigen expression on MP was analyzed by double-labeling with annexin V-FITC and PE-conjugated Mabs. The annexin V-FITC-positive MP were gated, and their binding of PE-labeled Mabs was evaluated in SSC vs. PE – fluorescence logarithmic plots.

Abbreviations

AGTII, angiotensin II; BSA, bovine serum albumin; CHX, cycloheximide; CPT, camptothecin; DMSO, dimethyl sulfoxide; FITC, fluorescein isothiocyanate; HBSS, Hanks' balanced salt solution; HUVEC, human umbilical vein endothelial cells; IC-IgG, IgG isotype control; Mab, monoclonal antibody; MP, microparticles; PE, phycoerythrin; PI, propidium iodide; PMA, phorbol-myristate-acetate; PS, phosphatidylserine; TRAP, thrombin receptor activating peptide (SFLLRNP); TNF, tumor necrosis factor; TPA, tissue plasminogen activator; TUNEL, terminal deoxynucleotidyltransferase dUTP nick end labeling; ZVAD, Z-Val-Ala-Asp-fluoromethyl ketone.

Authors' contributions

Author JŠ participated in the design of the study and carried out tissue culture and flow cytometry experiments. Author KH participated in flow cytometry experiments and in the design of the study. Author JGV participated in the design of the study and in its coordination.

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