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The release of microparticles by Jurkat leukemia T cells treated with staurosporine and related kinase inhibitors to induce apoptosis

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Abstract

Microparticles (MPs) are small membrane-bound vesicles released from cells undergoing activation or cell death. These particles display potent biological activities that can impact on physiologic and pathologic processes. Previous studies with the Jurkat T leukemia cell line demonstrated that staurosporine (STS) induces the release of MPs as cells undergo apoptosis. To investigate further this process, we tested the effects of STS, its analogue, 7-hydroxystaurosporine (UCN-01), and other protein kinase C (PKC) and cyclin-dependent kinase (CDK) inhibitors. FACS analysis was used to assess MP release. Results of these studies indicate that STS and UCN-01 induce MP release by Jurkat cells; in contrast, other PKC and CDK inhibitors failed to induce comparable release, suggesting that release does not result from simple inhibition of either kinase alone. Time course experiments indicated that STS-induced particle release occurred as early as 2 h after treatment, with the early release MPs displaying low levels of binding of annexin V and propidium iodide (PI). Early-release MPs, however, matured in culture to an annexin V- and PI-positive phenotype. Together, these results indicate that STS and UCN-01 induce MPs that are phenotypically distinct and reflect specific patterns of kinase inhibition during apoptosis.

Keywords

Microparticles; Apoptosis; Protein kinase C; Cyclin-dependent kinase; Staurosporine

Introduction

Microparticles (MPs) are small membrane-bound vesicles that are released from cells during the course of cell activation or death. These particles incorporate nuclear and cytoplasmic constituents and represent a novel class of extracellular organelles that can mediate intercellular regulation and communication [1–3]. Once released from cells, MPs have potent biological activities that can operate systemically as well as locally. These activities include immune modulation, coagulation and information transfer and may impact on a wide range of diseases including vascular, inflammatory and malignant conditions [4–8]. In

addition to their roles in disease pathogenesis, MPs can serve as biomarkers, representing remnants of apoptotic or activated cells in the blood that can be identified and enumerated by cell cytometry [3,9–15].

In elucidating the functional role of MPs in normal and pathologic settings, an important issue concerns the molecular properties of particles as they affect in vivo activity and detection in blood and other biological fluids. In general, flow cytometry is used to measure and quantify particles on the basis of size and display of cell surface markers. Since MPs arise from the cell membrane, they bear surface markers of their cell of origin. In addition, MPs display phosphatidylserine (PS), a membrane phospholipid that is usually on the inside of the cell membrane. PS can translocate to the surface during the course of apoptosis as well as cell activation because of effects on a trio of enzymes called flippase, floppase and translocase [3]. Once on the surface, PS can bind annexin V [16].

In previous studies, we used the Jurkat T leukemia cell line as a model and demonstrated that these cells, when treated with a variety of chemotherapeutic agents to induce apoptosis, released MPs [17]. As shown by flow cytometry, these particles bound annexin V, indicative of cell surface exposure of PS. In addition, these particles bound propidium iodide (PI) and contained RNA and DNA, consistent with translocation of nucleic components during the death process. Of agents tested, staurosporine, a prototypic protein kinase C inhibitor, showed the highest activity.

To extend these findings on MP generation and particle phenotype during apoptosis, we have characterized particle release from Jurkat cells treated in vitro with staurosporine (STS), as well as related protein kinase C (PKC) and cyclin-dependent kinase (CDK) inhibitors. We have focused on these agents because of the potency of STS in inducing cell death and particle generation from Jurkat cells and interest in the use of kinase inhibitors in the treatment of malignancy, including lymphoid tumors [17–20]. In results presented herein, we show that STS and its analogue 7-hydroxystaurosporine (UCN-01) but not other PKC or CDK inhibitors can stimulate the release of conventional annexin V- and PI-positive MPs during apoptosis as well as a phenotypically distinct group of MPs whose production occurs as early as 2 h after treatment. These early MPs are annexin V low or negative and have low binding of PI. With subsequent culture, however, these MPs can become annexin V-positive and bind PI. Together, results of these studies indicate the heterogeneity in the properties of MPs released from cells undergoing apoptosis and the ability of MPs to change phenotype over time.

Materials and methods

Cell culture and treatment

Jurkat cells were obtained from the Duke University Comprehensive Cancer Center Cell Culture Facility and were grown in Complete Medium (CM) composed of RPMI 1640 (Gibco BRL, Carlsbad, CA) supplemented with 10% heat inactivated fetal bovine serum (FBS) (Hyclone, Logan, UT) and 20 µg/ml gentamicin (Gibco). Cells in mid-logarithmic growth were harvested and pelleted by centrifugation at 400g for 5 min. The cell pellet was then resuspended at a density of 10^7 cells/ml in CM and cultured at 37°C in 5% CO₂. Apoptosis was induced by treating 4×10^7 Jurkat T cells with etoposide (10 µM), camptothecin (10 µg/ml), 7-hydroxystaurosporine (UCN-01) (5 µM) or staurosporine (STS) (1 µM) (all from Sigma–Aldrich Co., St. Louis, MO). Treated cells were incubated for times indicated. The media were collected and used for analysis of microparticles by flow cytometry as described below.

The CDK inhibitors, roscovitine, olomoucine II, and purvalanol A (concentration range: 0.01–10 μM) as well as the PKC inhibitors, bisindolylmaleimide, Gö 6983 and Gö 6976 (all from EMD Chemicals, Inc., Gibbstown, NJ and used in a concentration range of 0.008–25 μM) were also tested for induction of apoptosis and microparticle production. The pan-caspase inhibitor *N*-benzyloxycarbonyl-Val-Ala-Asp (*O*-methyl)-fluoromethyl ketone (Z-VAD-fmk; 100 μM) (Sigma–Aldrich Co.) was used to block caspase-dependent processes. A two-tailed *t*-test for unpaired samples with unequal variances was used for statistical analysis of MP numbers.

Isolation and characterization of microparticles

Microparticles were isolated from the culture using a two-step differential centrifugation process at room temperature. An initial centrifugation at 400g for 5 min was used to pellet out the treated cells. The cell-free supernatant was then centrifuged at 16,000g for 30 min in a microcentrifuge (Denville 2600, Denville Scientific, Inc., Metuchen, NJ) to isolate the MP pellet. MP pellets were resuspended in phosphate buffered saline (PBS) (Gibco) or Complete Medium (CM) at one-tenth the original volume of the treated cell culture. The MPs were then quantified and characterized by flow cytometry analysis (described below). MPs resuspended in CM were incubated further at 37°C with or without a caspase-inhibitor (Z-VAD-fmk; 100 μM) or 1 μM STS to test effects of subsequent incubation and caspase activity on phenotype.

In these experiments, control MPs were prepared from untreated Jurkat cells cultured in CM for 2, 18 or 2 h intervals following transfer into fresh media. For the 8 h time course experiment on particle release, Jurkat cells were centrifuged twice at 400g for 5 min, the supernatant containing MPs was discarded and the cells were plated in fresh CM in six-well plates (Cellstar, Greiner Bio-One, Munroe, NC). Cells cultured at a concentration of $10^7/\text{ml}$ for 2 h, were then centrifuged at 400g for 5 min to remove MPs released in the supernatant. The cell-free supernatant was further centrifuged to isolate MPs for assay. The cells were washed once in fresh CM that was equilibrated in a 5% CO_2 incubator at 37°C for 2 h and then resuspended in CM for continued culture. At 2 h intervals thereafter for the following 6 h, the procedure was repeated, with cells centrifuged to remove MPs which were assayed by flow cytometry. MPs from STS-treated Jurkat cells were similarly assayed to assess phenotypes at the different time intervals.

To assess nucleic acid content, MPs were first fixed and permeabilized using the BD Cytofix/Cytoperm Kit (BD Biosciences, San Jose, CA) as per the manufacturer's instructions. Briefly, the MP pellet isolated from 10^7 Jurkat cells was directly resuspended in 250 μl of BD Cytofix/Cytoperm™ solution in a microfuge tube and incubated at 4°C for 20 min. MPs were then pelleted at 16,000g for 30 min and washed twice in 500 μl of the BD Perm/Wash™ buffer containing a permeabilizing agent (saponin) and resuspended in 100 μl of the same buffer. Hundred-microliter of the enriched and permeabilized MP suspension was treated with 100 U/ml of RNase-free DNase (Invitrogen Co., Carlsbad, CA) or 200 U/ml of DNase-free RNase (Sigma–Aldrich Co.) or both and incubated at 37°C for 2 h to test the nucleic acid composition. MPs were analyzed using flow cytometry after staining with PI.

Flow cytometric analysis

MPs were counted and analyzed on a BD FACScan flow cytometer using BD CellQuest PRO software (BD Biosciences). A fifty-microliter aliquot of the treated cell culture or untreated control was diluted to 400 μl in Annexin-binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM CaCl_2) and analyzed on the flow cytometer to assess degree of cellular apoptosis by dual staining with PI (Sigma–Aldrich Co.) and fluorescein

isothiocyanate (FITC)-labeled annexin V (BD Biosciences). The MP suspension was prepared as described above at ten times the concentration in the original cell culture by resuspending in 1/10th the volume of PBS or CM. Fifty-microliter of this MP suspension was diluted to a final volume of 400 μ l in diluent containing PI or FITC-annexin V before analysis by the flow cytometer. MPs were diluted in 345 μ l of either calcium (Ca^{++}), magnesium (Mg^{++}) free phosphate buffered saline (PBS) and stained with 5 μ l of PI (nucleic acid stain) or annexin-binding buffer with 5 μ l FITC-annexin V to detect surface exposure of phosphatidylserine (PS). Data were collected in logarithmic mode at all channels. Sample buffer alone was used as a blank to set a side scatter threshold to exclude any noise. Analysis was carried out using FlowJo flow cytometry analysis software (Tree Star, Inc., Ashland, OR).

To determine MP numbers in the cell culture, the sample flow rate was first calibrated by weighing sample tubes before and after each timed run and averaging the differences. This sample flow rate was multiplied by the amount of time taken for each measurement to determine the total volume sampled. The final MP concentration was calculated by dividing the total counts measured by the total volume sampled and further multiplying by the dilution factors used in preparing the sample solution loaded on the flow cytometer.

Results

The induction of microparticle release by staurosporine

In these experiments, we have used the Jurkat human T cell leukemia line as a model to study the release of MPs from cells treated with protein kinase inhibitors to induce apoptosis. To measure MPs, we used flow cytometry and assessed particle number on the basis of light scatter. As demonstrated in previous studies [17], STS causes much greater particle production than does treatment with other agents such as camptothecin or etoposide when assessed at both early times and late times after the onset of treatment (Fig. 1). These findings confirm the activity of STS in MP release.

To investigate MP release by STS, a dose response and time course study was performed in the initial 2 h of treatment. As shown in Fig. 2a, while MP numbers remained low in cultures from untreated control cells, treatment of cells with increasing doses of STS led to a rapid increase in MP numbers, with highest levels observed with STS at 1 and 10 μ M. In these cultures, an increase in MPs began as early as 20 min after treatment and continued to increase thereafter. To correlate MP release with events in the cells, flow cytometry was performed using annexin V and PI as markers of stages of cell death. The number of cells with annexin-V binding but low PI binding was significantly increased in STS-treated cultures even at 2 h post-treatment (Fig. 2b).

The release of MPs early after treatment with STS is unexpected since particle release in many systems has been considered to occur late in apoptosis, at a time when blebbing and the formation of apoptotic bodies occur [21,22]. Furthermore, in some systems, STS can prevent blebbing, a process related to MP release [23]. In contrast, in our experiments, the MP release started rapidly after treatment with a PKC inhibitor which can nevertheless induce apoptosis. Since it is possible that some cells, depending on the stage in their cycle, undergo a rapid death with STS treatment, the relationship between apoptosis and MP production was further explored using a pan-caspase inhibitor. For this purpose, 4×10^7 Jurkat cells were pre-incubated for 1 h in medium containing 100 μ M Z-VAD-fmk, a pan-caspase inhibitor. The cells were then treated with STS and MP numbers determined at the end of 2 h. Whereas STS-treated cells released $3,948 \pm 267$ particles, cells pre-incubated with Z-VAD-fmk followed by STS released $4,376 \pm 359$ particles. This difference was not statistically significant ($P = 0.173$). In these experiments, Jurkat cells pre-incubated with Z-

VAD-fmk before treatment with STS showed significantly fewer apoptotic cells after 2 h than cells treated with STS alone and resembled the untreated control cells (data not shown). These findings indicate that the early release of MPs is caspase-independent.

The effect of PKC and CDK inhibitors on microparticle release

STS is a protein kinase C (PKC) inhibitor with a broad spectrum of action. In addition, it can inhibit other kinases including cyclin-dependent kinases (CDKs) [24]. To determine the target of STS action in inducing MP release, we tested related drugs as well as other inhibitors with similar specificity [25–28]. We first tested the ability of 7-hydroxystaurosporine (UCN-01), an analogue of STS that can also inhibit PKC, to induce MP production by Jurkat cells. Like STS, UCN-01 induced the early release of MPs although the dose–response of the two compounds differed (Fig. 3a).

In contrast to the findings with UCN-01, the PKC inhibitors, bisindolylmaleimide, Gö 6976 and Gö 6983 showed no significant change in MP counts at concentrations up to 1 μ M after 2 h (Fig. 3b). A slight increase in MP numbers was observed at the highest concentration tested (10 μ M), although this number of MPs was less than twice that of constitutively released MPs from untreated cells. To define further the target of STS action, CDK inhibitors, roscovitine, olomoucine II and purvalanol-A, were also tested for microparticle production. Figure 3c shows, among these CDK inhibitors, none induced significant MP production even at concentrations as high as 25 μ M. To determine whether these PKC and CDK inhibitors induced apoptosis of Jurkat cells under the conditions tested, flow cytometry was performed on cells cultured for 2 h. As these results indicate (data not shown), the treated cells showed a similar frequency of annexin V positive cells as did the untreated controls cells. Together, these findings indicate that, among agents tested, only STS and its analogue UCN-01 induced particle release in the early time-frame, indicating a response that cannot be explained by inhibition of either PKC or CDK alone.

Phenotypic properties of early microparticles

As defined by flow cytometry, MPs can be identified by their display of membrane phosphatidylserine, using annexin V for their identification and quantitation [1–3]. Furthermore, as shown by studies from this laboratory, particles from Jurkat cells arising in late apoptosis can bind PI [17]. PI binding is consistent with their content of nucleic acids and origin from cells in which membrane permeability is disturbed. Since the particles from STS-treated culture are produced after only 2 h, we investigated further the phenotype of the early particles, comparing them to constitutive MPs arising in untreated cultures as well as MPs obtained after 24 h at a time when apoptosis is extensive.

As shown in Fig. 4, flow cytometric analysis of the MPs produced within the first 2 h following treatment (early-release) with STS demonstrated a distinct phenotype that differed from that of particles produced constitutively by untreated control cell cultures or late in culture with STS. Thus, the early-release MPs showed low or absent binding of both annexin V as well as PI compared to MPs isolated from the untreated control cell cultures (Fig. 4a, b). These findings indicate that particles had an intact membrane as well as preservation of membrane asymmetry with PS confined to the inner membrane. As such, the early MPs differ from conventional MPs that bind annexin V because their membrane has “flipped,” and PS has translocated to the outer membrane leaflet; the binding of annexin V could also reflect permeability of MPs and the ability of annexin V to access internal PS.

To determine whether the phenotype of early MPs is stable following their release, particles were harvested by centrifugation after 2 h and then re-cultured in fresh medium for an additional 2 h. The phenotype of particles was then assessed by flow cytometry. Figure 4c, d

show the results of these experiments. Thus, after incubation in CM for 2 h at 37°C, the MPs bound FITC-annexin V and PI more strongly than when first released from cells. After 2 h of culture, these annexin V-positive and PI-positive MPs showed a similar phenotype as did the MPs isolated from untreated cell cultures (Fig. 4c, d); the binding of annexin V and PI was stable over that time interval. These findings indicate that, following release from cells, MPs can undergo phenotypic transformation, with changes in both membrane permeability and phospholipid distribution.

In these experiments, the yield of MPs from cultures treated with STS for 2 h was much greater than that from control, untreated cultures. Nevertheless, it is possible that even cells from control cultures could produce MPs with a phenotype characterized by low or absent binding of annexin V and PI. Therefore, to assess whether MPs with this distinct phenotype were present in untreated cells, a different experimental format was used to obtain a population of control MPs. For this purpose, control cells were washed twice and resuspended in fresh media, harvesting MPs after 2 h as opposed to a longer culture time. Using this type of preparation, the presence of MPs with low or absent binding of annexin V and PI was also observed in untreated cell cultures when sampled at 2 h (Fig. 5a). This finding demonstrates that this distinct MP phenotype may also be constitutively expressed, although in much lower numbers than that from cells treated with STS. Consistent with a maturation process observed with MPs from cells treated with STS or UCN-01, MPs isolated from control cell culture showed increased annexin V and PI binding as culture progressed (Fig. 5a, top panel).

To study the effects of STS on the maturation process of MPs released from control, UCN-01 and STS-treated cultures, MP populations from all three types of culture were isolated by centrifugation and resuspended in fresh media containing 1 μ M STS or media without STS. In these experiments, MPs released from UCN-01-treated and STS-treated Jurkat cells transitioned to an annexin V and PI-binding state irrespective of whether they were incubated in medium with or without STS (Fig. 5a, middle and bottom panels).

The MPs obtained from the control, untreated cells showed a somewhat different pattern of response. Whereas these MPs showed greater annexin V binding with culture in media alone, incubation with 1 μ M STS showed a greater increase in annexin V binding than that of MPs incubated in Complete Medium alone. In contrast, these particles showed a transition to higher PI binding independent of the presence of STS in the culture (Fig. 5a, top panel). Together, these findings indicate that, while the generation of the MPs with low or absent annexin V and PI binding may not require STS, STS can nevertheless influence the maturation process to an annexin V-positive state.

In further time course experiments to characterize the phenotypic progression of MPs, MPs released during 2 h intervals were obtained from cultures of cells and analyzed by FACS. For this purpose, at consecutive 2 h intervals, MPs from the preceding period of culture were obtained by centrifugation and analyzed; this procedure was followed to reduce the presence of MPs that may have transitioned to an annexin V- and PI-positive state during a more extended culture period. In these experiments, analysis of MPs isolated from STS-treated Jurkat cells indicated that MPs that are released over time following treatment are increasingly annexin V- and PI-positive (Fig. 5b, bottom panel). In contrast, particles from control, untreated cells showed a more consistent phenotype, although, at later time points, MPs with some increase in either annexin V or PI binding were observed (Fig. 5b, top panel). These findings further support an influence of STS on the phenotype of MPs, an effect that may reflect events in both the cells and the MPs.

Since many events during apoptosis including membrane changes are caspase-dependent, the effects of the pan-caspase inhibitor on the maturation of early-release MPs was tested. For this purpose, the early-release MPs were harvested from the cultures and then incubated in CM containing 100 μ M Z-VAD-fmk prior to analysis by flow cytometry. Even in the presence of this inhibitor, the MPs underwent the transition to an annexin V and PI-binding phenotype (Fig. 6). Thus, both the release of particles from STS-treated cells and their subsequent phenotypic transformation are independent of caspase activity.

The binding of PI indicates that early release MPs contain nucleic acid and are similar in this respect to MPs released during apoptosis. As PI binds both DNA and RNA, identification of the nucleic acid present requires subsequent analysis, with sensitivity to DNase and RNase allowing biochemical distinction. In a previous study, we showed that MPs obtained from STS-treated cells contained both DNA and RNA and that PI binding in fixed and permeabilized particles was sensitive to both DNase and RNase [17]. We also confirmed this finding by purifying and measuring the DNA and RNA content by biochemical assay. This prior study provides the basis for using nuclease sensitivity as an assay for measuring the relative content of DNA and RNA.

To characterize the nucleic acids present in the different classes of MPs, preparations from untreated and STS-treated cells were harvested, fixed and permeabilized, and then treated with either RNase or DNase prior to PI staining. Figure 7 presents these results. As these findings show, DNase or RNase treatment of permeabilized microparticles isolated from the untreated culture reduced PI binding. Combined treatment with both nucleases abrogated PI binding (Fig. 7, top panel). In contrast, the microparticles isolated from the STS-treated cultures showed a marked reduction in PI binding after RNase treatment but almost no reduction in PI binding following DNase treatment. Treatment with both RNase as well as DNase abrogated PI binding (Fig. 7, bottom panel); the difference between MPs treated with both nucleases and those treated with RNase alone was less significant than that observed in MPs from control cell cultures (Fig. 7, top panel). These findings indicate that early release MPs from STS-treated cells contain primarily RNA and therefore differ from those constitutive particles which contain both DNA and RNA.

Discussion

Results presented herein provide new insights into the release of cellular MPs in cells treated with PKC and related CDK inhibitors as well as the kinetics and phenotypic variability of MP production. Thus, we showed that Jurkat T cells treated with STS or the analogue UCN-01 demonstrated time-dependent release of MPs, including a population with low levels of binding of annexin V as well as PI; release of these MPs occurred rapidly within 2 h of treatment, these MPs were also present in control cultures, albeit at much lower numbers. This phenotypic pattern (i.e., low annexin V and PI binding) implies an intact membrane and the asymmetric distribution of PS localization similar to that of a living cell [29,30]. Furthermore, our studies show that, with subsequent culture, these particles undergo an extracellular maturation process to become annexin V-positive and PI-positive. Together, these observations indicate that particle release from cells treated with certain PKC and CDK inhibitors to induce apoptosis is dynamic and heterogeneous and that, following release from cells, MPs can undergo membrane events that alter their phenotype.

As shown in studies of normal and malignant cells in vitro, the release of MPs occurs in two main settings, cell activation and cell death [31]. While activated cells can release particles, the basis of this process has not been extensively studied and may vary depending on cell type. In contrast, during apoptosis, particle release may be related to blebbing, a process which causes the formation of small bubble-like extrusions at the membrane in association

with the translocation of both nuclear and cytoplasmic contents [22,32,33]. Blebbing can occur both early and late in apoptosis, although the occurrence may differ with cell lines that are attached and those that grow in suspension. In addition to particle release by blebbing, the formation of apoptotic bodies during the course of apoptosis may produce cell fragments that phenotypically can also be characterized as MPs. Since the definition of MPs depends on size, operational distinction between blebs and apoptotic bodies by flow cytometry may not be possible.

During apoptosis, this blebbing process depends on the ROCK enzyme and is associated with changes in the cytoskeleton [34,35]. While the surface of the apoptotic cell can be covered with blebs, only a limited number is actually released [36]. In contrast, depending on the cell type, the formation of apoptotic bodies may be more extensive, contributing to the production of extracellular particulates. While MP formation may reflect more than one mechanism, nevertheless, particle release may be important to disease pathogenesis because of the immunological activity of MPs as well as their prothrombotic activity resulting from their surface display of tissue factor and PS [14,37].

MPs may exert physiological and pathological activities beyond thrombosis and immune modulation. Thus, as shown in in vitro experiments on a novel mechanism of intercellular communication, MPs can transfer genetic information because of their content of DNA and RNA, including regulatory microRNA [38–40]. Furthermore, MPs from leukemia cells among other cell types can transfer cell surface receptors and thereby modify the response of recipient cells [41]. This phenomenon may occur during the course of leukemia as well as other malignancies and represent a mechanism by which malignant cells can modify their microenvironment or perturb responses of nearby non-malignant cells [2].

As our studies indicate, treatment of Jurkat cells with STS or UCN-01 caused significant MP production, with the majority of the MPs released within the first 2 h of treatment although the phenotype of these MPs varied depending on the time of sampling. Thus, the MPs released within 2 h showed low or absent binding of both annexin V and PI. This pattern differs from that of MPs obtained from cultures after 18 h of treatment as well as MPs from untreated cultures. Like MPs from cultures treated with agents to induce apoptosis, MPs from control cultures were annexin V- and PI-positive. In the case of 18 h old untreated cultures, MPs may originate from cells undergoing spontaneous apoptosis since, as shown previously, Jurkat cells in cultures usually contain an appreciable percentage of apoptotic cells [17]. In addition to MP release during apoptosis, spontaneous particle release may reflect a shedding process in which cells discharge membrane and macromolecular contents as part of their growth. This shedding has been observed in cells treated with ionomycin, ATP or PMA and may be a facet of a process termed enlargeosome exocytosis [42].

While STS stimulates the release of annexin V low, PI low MPs, such MPs were also present in the media of control cells, although their numbers were limited. To identify this population, media were obtained from cells cultured for short periods of time after transfer to fresh media; this procedure was intended to reduce the number of particles which accumulate during prolonged culture at which point they may represent the preponderant particle type. Importantly, as in the case of MPs from STS-treated cells, additional culture of these MPs from control cells obtained after 2 h demonstrated a transition to a phenotype with increased binding of annexin V and PI. Thus, these studies indicate that phospholipid exposure to allow annexin V binding may reflect events subsequent to the release of a particle from a cell. Similarly, breakdown in membrane permeability may occur in MPs after detachment from a cell still undergoing apoptosis.

This maturation of particles includes changes expected for apoptosis (i.e., PS exposure), although it was not blocked by a caspase inhibitor. These findings indicate that MPs themselves are in a dynamic state and can undergo changes associated with apoptosis, including membrane events. As these considerations indicate, the phenotype of MPs could reflect more than one mechanism. Thus, MPs, obtained from treated or untreated cells, could show an annexin V, PI positive phenotype because they were released by cells whose membrane changed during apoptosis. In this case, the phenotype of the released particles would resemble that of the apoptotic cells.

In another scenario, the annexin V, PI positive phenotype could reflect the maturation of a population of MPs released early after culture, with or without STS treatment that originally had the low binding phenotype. Eventually, in culture, this population would mature to have high binding of both annexin V and PI. In this regard, as our experiments show, STS may affect the maturation process since MPs obtained from control cultures showed a more rapid maturation when treated with STS than MPs cultured in media alone. Thus, STS could affect both the process of particle release and the phenotype of particles after release. The majority of the release process occurs during the first 2 h of culture, with particle release decreasing subsequently.

While the appearance of PS on the membrane has usually been considered the result of phospholipid translocation during apoptosis, another possible mechanism for the externalization of PS relates to the integration of endosomal membrane components. In this scenario, in the presence of high Ca^{2+} , internal membranes transit to the external membrane as a membrane repair process [43]. Thus, whether PS results from membrane flipping or membrane replacement, it is possible that membrane events during apoptosis, either spontaneous or secondary to STS treatment, are not uniform and that the released particles originate from parts of the membrane that have retained their phospholipid orientation and membrane integrity.

Staurosporine is a general inhibitor of protein kinases and can induce apoptosis by more than one mechanism, with caspase-dependent and independent pathways operative. Because its effects on kinases extend beyond PKC and CDK, STS can affect inhibition of diverse phosphorylation events including those that regulate microfilament assembly and cytoskeletal organization [44,45]. As shown in prior studies, these changes are caspase-independent. While these changes have been defined primarily in terms of cell morphology and cytoskeletal organization, it is possible that they lead to MP release. In this regard, because STS can affect the cell cytoskeleton as well as inhibit blebbing in certain settings [23,44], MP release (both early and late) during treatment with this agent may differ from that occurring during apoptosis induced by other treatments. In addition, while STS may affect blebbing, nevertheless, cells treated with this agent may release apoptotic bodies which can be phenotypically characterized as particles.

In addition to identifying a novel class of MPs, these studies have defined the pharmacological basis for their expression using agents that have been explored for the treatment of human malignancy either alone or in combination with other drugs [20,26,27,46,47]. In view of previous studies on MP release by Jurkat cells, we focused on inhibitors of PKC and related kinases. Among compounds tested using Jurkat cells, STS caused the most robust production of particles at 2 h. Staurosporine is a broad inhibitor of protein kinases and does not exhibit specificity for particular PKC isozymes [24,48]. While STS may compete with ATP to inhibit kinase action, its precise mode of action is not well defined.

Our studies on other PKC inhibitors demonstrated that UCN-01 is also active in early particle release but that other PKC inhibitors differing in chemistry as well as specificity failed to induce early MP release. Furthermore, inhibitors of related CDKs, which are also blocked by STS and UCN-01, also failed to induce early particle release. Together, these studies suggest that mechanisms of MP release, while resulting from kinase inhibition, may be complex and depend on various kinases present in the cell. The broad action of existing inhibitors limits identification of the relevant target enzymes.

Given the increasing attention to the study of MPs as an extracellular organelle mediating events during disease pathogenesis, these findings have implications in a number of distinct areas clinically. Thus, the demonstration of phenotypic heterogeneity of MPs indicates the need for assays that can measure MP numbers in the blood and other biological fluids more completely, expanding biomarker approaches beyond annexin V binding. Second, our findings suggest that particle release by agents used as chemotherapeutics may reflect cellular events in addition to activation and death and may occur early as well as late in the use of protein kinase inhibitors. Finally, since STS analogues are being tested in a variety of malignancies [19,24,26,46,47], an assessment of MP phenotype and release in vivo and in vitro may provide a new way to evaluate the cytotoxic effects of these agents and provide a useful biomarker that can be readily obtained from blood samples.

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Fig. 1.

Induction of Jurkat cell microparticles release. 4×10^7 Jurkat cells were treated with staurosporine (STS, 1 μ M), camptothecin (CAM, 10 μ g/ml), or etoposide (ETO, 10 μ M) for 2 h (*shaded*) and 18 h (*unshaded*). MPs were isolated from cell culture by differential centrifugation as described in “Materials and methods” and counted by light scatter by flow cytometry

Fig. 2.

Time course of microparticle release from STS-treated Jurkat cells. **a** presents the time-course of MP production from 4×10^7 Jurkat T cells in cultures treated with various concentrations of STS (10 nM–10 μM). **b** presents analysis of PI binding and annexin V binding of cells at 2 and 18 h after treatment. The plots indicate the proportion of live cells (annexin V-negative, PI-negative), early apoptotic cells (annexin V-positive, PI-negative) and dying cells (annexin V-positive, PI-positive). Untreated control cells were treated with equal volume of diluent (DMSO) without inhibitors. Experiments were performed in triplicate and repeated at least thrice

Fig. 3. Effect of PKC and CDK inhibitors on Jurkat MP production. In these experiments, 2×10^7 Jurkat cells were treated with various concentrations of PKC or CDK inhibitors and numbers of MPs determined by flow cytometry. **a** Effect of STS (closed diamonds) or its analogue UCN-01 (open diamonds) (8 nM–25 µM) on Jurkat MP production; **b** Effect of PKC inhibitors on Jurkat MP production. Results are presented for bisindolylmaleimide (circles), Gö 6976 (triangles), Gö 6983 (squares) and STS (diamonds); **c** Effect of CDK inhibitors on Jurkat MP production. Results are presented for roscovitine (squares), purvalanol A (triangles), olomoucine II (crosses) and UCN-01 (open diamonds). Untreated control cells were treated with equal volume of diluent (DMSO) without inhibitors. Experiments were performed in triplicate and repeated at least thrice

Fig. 4. Membrane properties of MPs from STS-treated and untreated Jurkat cells. Flow cytometric analysis of STS-induced MPs (solid line) and control MPs (dotted line) stained with FITC-annexin V (a) or PI (b). c, d Presents a comparison of annexin V binding (c) and PI binding (d) of MPs before (solid line) and after (dotted line) a 2 h incubation in CM. The top panels indicate MPs obtained from control cultures (Con) while the bottom panels indicate particles obtained after 2 h of treatment with STS. As these results indicate, after culture, MPs obtained after an initial 2 h of STS treatment increased binding of both PI and annexin V, indicating a change in membrane properties. Histograms are representative of at least four replicates

Fig. 5.

The phenotype of MPs in culture. **a** Flow cytometric analysis of MPs released early (2 h of culture) from untreated control (*top panel*), STS-treated (*middle panel*) and UCN-01-treated (*bottom panel*) Jurkat cells. MPs isolated from each Jurkat cell culture (*dotted line*) were incubated for an additional 2 h at 37°C and 5% CO₂ in fresh, equilibrated Complete Medium only (*solid line*) or in the presence of 1 μM STS (*broken line*) to assess changes in annexin V and PI-binding. **(b)** MPs were isolated from STS-treated (*bottom panel*) and untreated control (*top panel*) Jurkat cell cultures at 2 h intervals for a total of 8 h following treatment. MPs were obtained from the cells by centrifugation at each 2 h time-point and isolated as described in “Materials and methods”. The MPs released at the intervals of 0–2 h (*dark grey*

peak), 2–4 h (*solid line*), 4–6 h (*broken line*) and 6–8 h (*light grey peak*) were assessed for annexin V- and PI-binding

Fig. 6.

Effect of caspase inhibitor on the change in MP membrane permeability. MPs from STS-treated cells (*dotted line*) were incubated with 100 μ M Z-VAD-fmk (pan-caspase inhibitor) for 2 h (*broken line*) or left untreated (*solid line*) in CM to test the influence of caspase activity on the transitioning of PI binding. Histograms are representative of four replicates. As these results indicate, the caspase inhibitor did not affect the increase in PI binding

Fig. 7. Nucleic acid content of microparticles. Nucleic acid composition of control (*top panel*) and STS (*bottom panel*) MPs isolated 2 h after the beginning of treatment was assessed by PI binding using treatment with 200 U/ml of DNase (*grey line*), 100 U/ml of RNase (*black line*), a combination of both (*broken line*) or left untreated (*dotted line*) to distinguish RNA and DNA content. Histograms are representative of at least four replicates