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Differential detergent sensitivity of extracellular vesicle subpopulations

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Abstract

Extracellular vesicles (including exosomes, microvesicles and apoptotic bodies) are currently attracting rapidly increasing attention from various fields of biology due to their ability to carry complex information and act as autocrine, paracrine and even endocrine intercellular messengers.

In the present study we investigated the sensitivity of size-based subpopulations of extracellular vesicles to different concentrations of detergents including sodium dodecyl sulphate, Triton X-100, Tween 20 and deoxycholate. We determined the required detergent concentration that lysed each of the vesicle subpopulations secreted by Jurkat, THP-1, MiaPaCa and U937 human cell lines. We characterized the vesicles by tunable resistive pulse sensing, flow cytometry and transmission electron microscopy.

Microvesicles and apoptotic bodies were found to be more sensitive to detergent lysis than exosomes. Furthermore, we found evidence that sodium dodecyl sulphate and Triton X-100 were more effective in vesicle lysis at low concentrations than deoxycholate or Tween 20.

Taken together, our data suggest that a combination of differential detergent lysis with tunable resistive pulse sensing or flow cytometry may prove useful for simple and fast differentiation between exosomes and other extracellular vesicle subpopulations as well as between vesicular and non-vesicular structures.

Introduction

Extracellular vesicle (EV) is a collective term for membrane-enclosed structures secreted by cells in a constitutive or induced manner or by apoptotic cells. To date EVs have been found to be secreted by cells of a vast diversity ranging from bacteria to human cells (1, 2).

A significant attention is currently devoted to the applications of EVs in many areas of biology given their potential as novel biomarkers, therapeutic targets or tools. Although the field is experiencing very rapid growth, there is still no consensus on the classification of these structures. Until now no clear evidence is available for the exclusive association of molecular markers with specific EV subpopulations generated by different biogenesis pathways. Our research group and others have provided some evidence that size fractionated EV subpopulations including exosomes (EXOs, ~100 nm in diameter), microvesicles (MVs, 100-800 nm) and apoptotic bodies (APOs, 1-5 μm) differ in several important parameters such as nucleic acid content, membrane lipid packing density, protein to lipid ratio and electron microscopic morphology (3-5).

Earlier data from our laboratory has shown that EVs can be differentiated from protein aggregates (such as immune complexes) by detergent treatment (0.05% Triton X-100) (6). This is based on the fact that lipid membrane enclosed vesicles are more sensitive to detergent lysis than protein aggregates. Although such detergent treatments to disrupt vesicular isolates have been used in different studies (7-11), to date there is no clear evidence as to which concentrations and detergents are optimal for lysing different EV subpopulations.

In this work we used different concentrations of the widely used anionic detergent sodium dodecyl sulphate (SDS), the non-ionic detergents Triton X-100 and Tween 20, and the mild biological detergent and constituent of bile, sodium deoxycholate.

Here we provide the first clear evidence for the required concentrations of the above detergents to lyse different EV subpopulations. Importantly, we also show that their use can be combined with either tunable resistive pulse sensing (TRPS) or flow cytometry and enables to confirm the vesicular nature and subpopulation of the detected structures.

Materials and Methods

Cell lines

Jurkat (TIB-152) human T cell lymphoma, THP-1 (TIB202) human acute monocytic lymphoma and U937 human histiocytic lymphoma cell line were obtained from ATCC. The MiaPaCa pancreatic cancer cells were kindly provided by Dr. Klaus Felix, (Universität Heidelberg, Heidelberg). The cell lines were cultured in RPMI medium containing 10% (v/v) fetal bovine serum, 2 mM glutamine, and 1% Antibiotic Antimycotic Solution (Ab/AM) (all from Sigma-Aldrich), at 37^{o}C in 5% CO_2/air.

EV isolation from cell culture supernatants

Cells (10^6/mL except for the adherent MiaPaCa cells that were grown to 60-70% confluence) were allowed to produce EVs for 24 hours in serum-free RPMI medium. Cell viability was >90-95% as confirmed by using Annexin V FITC and propidium iodide (both from BD Biosciences) staining as described before (3,12). EV-containing conditioned serum-free media was submitted to 300g centrifugation to deplete cells, and stored at -80^{o}C prior to analysis by TRPS.

For transmission electron microscopy and flow cytometry three different EV subpopulations were isolated including APOs, MVs, and EXOs by size filtration and differential centrifugation. Briefly, cells were removed by centrifugation at 300g for 10 min, and the supernatant was filtered by gravity through a 5µm filter (Millipore) and centrifuged at 2,000g for 10 min at room temperature to pellet APOs. Next, the supernatant was filtered by hydrostatic pressure through a 0.8µm filter (Millipore) and centrifuged at 12,600g for 30 min at room temperature to pellet MVs. Finally, the supernatant was ultracentrifuged at 100,000g for 70 min at 4^{o}C to pellet EXOs in an Optima MAX-XP ultracentrifuge with a MLA-55 rotor (Beckman Coulter).

Detergent treatment of extracellular vesicles

Detergents used in this study (sodium dodecyl sulphate, Triton X-100, Tween 20 and sodium deoxycholate) were purchased from Sigma-Aldrich. Detergents were added to EV-containing samples to different final concentrations (w/v for SDS and deoxycholate and v/v for Triton X-100 and Tween 20), vortexed for 30 seconds, and analyzed by TRPS or flow cytometry. All steps of detergent lysis were carried out at room temperature. These conditions were established based on preliminary experiments using different incubation times of EVs with detergents and showing essentially no difference in lysis efficiency with increasing time (data not shown).

Tunable resistive pulse sensing measurements

EV-containing cell-depleted conditioned media were submitted to TRPS analysis using qNano (IZON Science) as described previously (5, 13). At least 500 particles were counted (except when lysis was observed in which case one minute recording time was used regardless of particle counts). NP100, NP400, NP800 and NP2000 nanopore membranes stretched between 43-47 mm were used. Voltage was set in between 0.04-0.7 V to achieve a stable current between 115-145 nA. Particle-size histograms were recorded when root mean square noise was below 12 pA, and particle rate in time was linear using calibration beads CPC100B, CPC400E, CPC800D and CPC2000D (mode diameters 110nm, 340nm, 740nm and 1900nm, respectively) (all from IZON). At every detergent concentration step the calibration beads were also measured at least twice in order to detect any difference in their modal size and particle rate possibly due to the presence of detergent micelles. For none of the detergents this was found to be the case (data not shown).

Transmission electron microscopy

Representative EV pellets prepared by differential centrifugation/ultracentrifugation and gravity driven size filtration were fixed with 4% paraformaldehyde in 0.01M PBS for 60 min

at room temperature. The preparations were postfixed in 1% OsO_4 (Taab) for 30 min. After rinsing with distilled water, the pellets were dehydrated in graded ethanol, including block staining with 1% uranyl-acetate in 50% ethanol for 30 min, and were embedded in Taab 812 (Taab). Overnight polymerization of samples at 60°C was followed by sectioning, and the ultrathin sections were analyzed using a Hitachi 7100 electron microscope (Hitachi Ltd.) equipped with a Megaview II (lower resolution, Soft Imaging System) digital camera.

Flow cytometry of extracellular vesicles

EVs were incubated with Annexin V APC and an anti-CD9 FITC antibody (both from BD Biosciences) for 30 min at room temperature in the dark. Autofluorescence was detected in the absence of fluorescent Annexin V or anti-CD9. Fluorescence of the labelled antibody and Annexin V in the absence of EVs was also determined within the EV gates. EXOs were coupled to 4µm aldehyde/sulphate latex beads (Life Technologies), blocked with 1% bovine serum albumin in PBS for 2 hours at room temperature. Glycine (100mM) was used for further blocking. To verify the TRPS findings, APOs, MVs and EXOs (the latter bound to beads) were incubated with detergents at concentrations that have been shown to lyse vesicles by TRPS, and were measured by a FACSCalibur flow cytometer (BD Biosciences). Instrument settings and gates were set as described earlier (14, 15) using Megamix beads (BioCytex) optimized with 1 µm Silica Beads Fluo-Green (Kisker). Data were analyzed by FlowJo software (Treestar).

Statistical analysis

For data analysis we used GraphPad Prism v.6. For analysis of separate cell line-derived EV counts we used two-way ANOVA. For the analysis of combined EVs counts for all cells and all detergents, one-way ANOVA was used, and for parameters not showing normal distribution, we applied Kruskal-Wallis non-parametric test. P values <0.05 were considered statistically significant (* $P<0.05$, ** $P<0.01$ and *** $P<0.001$).

Results

Electron microscopic confirmation of the simultaneous presence of all three EV subpopulations in conditioned media of cell lines

EV-containing conditioned media of Jurkat, THP-1, U937 and MiaPaCa cells lines were collected for further analysis. Figure 1 shows a representative transmission electron microscopic image of pelleted EVs from conditioned media collected from THP-1 cells. As shown in the figure, a combination of all three EV size-based subpopulations (APO, MV and EXO) was simultaneously present in pellets of the conditioned media.

Size distribution analysis of EV subpopulations in the presence of different concentrations of detergents

We first wanted to study if the different concentrations of detergents had an effect on the size distribution of different EV subpopulations using TRPS. Different size-based subpopulations of EVs were analysed by different sized pores in the TRPS system. NP100 membranes with the detection range of 60-300 nm were used for the detection of EXOs. A combination of NP400 and NP800 was used for the analysis of MV-sized EVs (with a detection range 150-1000 nm). Finally, NP2000 with a detection range of 1000-5000 nm, was used for the detection of APO-sized structures in the conditioned media. Prior to TRPS analysis, cell-depleted conditioned media were supplemented with different concentrations of detergents, and were vortexed for 30 seconds.

We detected differences in the original concentrations of EV subpopulations prior to detergent lysis and found that the concentrations of EXOs, MVs and APOs were in the ranges shown in Supplementary Figure 1. Furthermore we found that the concentration of EVs released by each cell type differed significantly from one another (Supplementary Figure 1).

Our results did not show any shift in the size ranges of the individual EV subpopulations upon detergent treatment. Neither could we observe any significant deviation from the modal size of each EV subpopulation. Additionally, we did not find a correlation between the original concentration of unlysed EVs and the concentrations of the detergents required for lysis.

In Figure 2, 3, and 4 are shown representative size distribution histograms of APOs, MVs, and EXOs, respectively, as detected with increasing concentrations of SDS. SDS resulted in strong decrease in the EV concentrations. Similarly to SDS, the other three detergents (Triton X-100, deoxycholate and Tween 20) had no effect on the modal size and size ranges of individual EV subpopulations (data not shown).

Differential sensitivity of EV subpopulations to detergent lysis

Figure 5 summarizes data on the differential ability of the four applied detergents to disrupt EV subpopulations. All four detergents tested were found to lyse EVs albeit at different concentration ranges. Out of the four detergents, SDS was found to reduce APO and MV counts at the lowest concentrations (0.01%) while Triton X-100 lysed EXOs at the lowest concentration (0.075%). In contrast, Tween 20 was found to be the least effective (reducing EV counts at very high concentrations (10-15%) only.

EVs derived from the four different cell lines showed similar detergent sensitivity within each size-based subpopulation regardless of the original concentration of the secreted EVs present in the conditioned media. We found no statistically significant decrease in EV counts until the suggested lysing concentrations for each detergent. For all detergents, at these lysing concentrations we found statistically significant reduction of the measured EV counts (two-way ANOVA) of each separate cell line: $p < 0.0001$ for APOs, $p<0.05$-0.0001 for MVs (with the exception of SDS lysis of Jurkat MVs), and $p< 0.01$-0.0001 for EXOs. Clearly, EXOs were found to be the least sensitive to detergent lysis while APOs and MVs showed higher sensitivity to detergent lysis (and were similar to each other). Similarly to Triton X-100, the bile acid deoxycholate had moderate efficiency to disrupt all EV subpopulations.

Interestingly, SDS lysed EXOs at least at an order of magnitude higher concentration (0.125%) than what was required for lysing MVs and APOs (0.01%). In contrast, all three EV subpopulations resisted concentrations of Tween 20 as high as 5%.

Flow cytometric validation of TRPS findings

Finally, EVs were characterized with flow cytometry by their staining with fluorochrome labelled annexin V and anti-CD9 antibody (Figure 6). Annexin V and anti-CD9 stained all three EV subpopulations. Using SDS concentrations shown in the present study by TRPS to lyse vesicles, we could demonstrate the prompt disappearance of fluorescent events validating our prior TRPS observations. The lysing concentrations of Triton X-100 determined by TRPS were also verified by flow cytometry (data not shown).

Discussion

In the past decade EV research took an important stage not only in cell biology, but also in many other fields of biomedical research. The recognition that cells secrete lipid bilayer enclosed structures that have previously been overlooked and considered debris, has brought a new paradigm to biology from bacteriology to cancer biology and immunology among others. These subcellular structures have brought about the realization that cells can communicate by EVs in ways that have been previously thought possible only by individual molecules such as cytokines or hormones. However, despite of the plethora of novel functions and roles of EVs, this field has been held back by the scarcity of tools with which to characterize and validate the presence, purity, amount and subtype of EVs.

Based on previous findings of our group, in this work we carried out a comprehensive analysis of the effect of detergents on size-based EV subpopulations. This study provides a practical tool to confirm the vesicular nature of particles present in biological samples using either TRPS (used for enumeration and size distribution measurements of EVs) or flow cytometry. Given that both TRPS and flow cytometry are commonly used methods to characterize EVs, the use of detergent control in EV analysis may prove useful in a wide variety of experimental settings and may possibly be combined with other techniques of EV analysis as well.

Results of this study clearly demonstrate that SDS is the detergent that disrupts MVs and APOs at the lowest concentration. This is in good accordance with the fact that ionic detergents, such as SDS, are extremely effective in the solubilisation of membrane proteins (16). We also found that there is an order of magnitude difference between the concentration of SDS that lyses both APOs and MVs and the one that lyses EXOs. Therefore

the use of SDS for differential lysis of APOS, MVs and EXOs may appear superior to other detergents. However, for downstream applications it also has to be considered that in most instances ionic detergents are denaturing to some extent, while bile acids and non-ionic detergents are relatively mild and are usually non-denaturing (16). The choice of detergent for EV studies is thus, dictated by the type of work to be carried out. Non-ionic detergents are known to break lipid–lipid interactions and lipid–protein interactions rather than protein–protein interactions, a feature that enables them to differentiate EVs from protein aggregates (6, 17).

Our data point to the similarity of the lipid membrane composition of APOs and MVs as opposed to EXOs in line with our previous observations related to lipid membrane composition and membrane liquid order of different size-based EV subpopulations (5). Briefly, EXOs were found to be more liquid ordered while both APOs and MVs had similar and lower membrane liquid orders (5). This is in accordance with the findings that liquid ordered as opposed to liquid-disordered membranes are resistant to detergents (18, 19).

Finally, our findings may have impact on EV research beyond TRPS and flow cytometry. By using detergents at the suggested concentrations, vesicle enclosed cytokines, RNAs and metabolites may be released for detection. Also, analysis of vesicle cargo by Western blotting or mass spectrometry requires both the disruption of the vesicle membrane and the release of membrane proteins. Recently, particular attention follows RNA as a vesicular cargo. RNA extraction from EVs may involve the use of detergents (20). Results of our study also have important implications in other research methods such as in ELISAs where Tween 20 is used at lower concentrations (typically 0.05-0.1%) than what we found to lyse EVs.

In conclusion, for the first time our study compared the efficacy of different detergents to lyse EVs, and demonstrated that different size-based subpopulations of EVs secreted by highly diverse human cell lines showed similar detergent sensitivity patterns. Furthermore, this study determined the concentrations at which different types of EVs were lysed by detergents, and showed the feasibility of combining detergent lysis with the TRPS technique.

Taken together, our data validate the use of detergent lysis as a control and an integral component of the EV detection toolbox.

Legends for figures

Figure 1. Transmission electron microscopy of EVs in conditioned media

Serum-free, cell-depleted 24h conditioned medium of THP-1 cells was ultracentrifuged at 100,000g 60 min and submitted for electron microscopic imaging. Extracellular vesicles from all size-based subpopulations were present simultaneously. APO: apoptotic body, MV: microvesicle, EXO: exosome.

Figure 2. Size distribution of APOs in the presence of increasing concentrations of SDS

The figure shows representative TRPS-determined size distribution histograms of the APOs in 24h serum-free conditioned media of four different human cell lines in the absence or presence of increasing concentrations of SDS. Numbers in the size histograms indicate mean ± SD of total concentration of EVs/mL in cell-depleted conditioned media.

Figure 3. Size distribution of MVs in the presence of increasing concentrations of SDS

The figure shows representative TRPS-determined size distribution histograms of the MVs in 24h serum-free conditioned media of four different human cell lines in the absence or presence of increasing concentrations of SDS. Numbers in the size histograms indicate mean ± SD of total concentration of EVs/mL in cell-depleted conditioned media.

Figure 4. Size distribution of EXOs in the presence of increasing concentrations of SDS

The figure shows representative TRPS-determined size distribution histograms of the EXOs in 24h serum-free conditioned media of four different human cell lines in the absence or presence of increasing concentrations of SDS. Numbers in the size histograms indicate mean ± SD of total concentration of EVs/mL in cell-depleted conditioned media.

Figure 5. Comparison of detergent lysis sensitivity of EV subpopulations

TRPS was used to compare sensitivity of APOs, MVs and EXOs secreted by different cell lines (Jurkat, THP-1, U937 and MiaPaCa) to increasing concentrations of detergents (SDS, Triton X-

100, deoxycholate and Tween 20). Means ± SD of at least three independent measurements are represented. Given the magnitude differences in EV concentrations, 100% in the Y axis represents 100% of mean EV concentration prior to detergent treatment. The X axis shows the concentrations of the detergents used with red colour representing lysing concentrations. Note that the detergent concentration ranges differ due to the differences among detergents in working ranges for EV lysis. Combined EV counts for all cells were analyzed by one-way ANOVA or Kruskal-Wallis test (for parameters of non normal distribution). **p <0.01; *** p<0.001

Figure 6. Flow cytometric validation of EV lysing concentration of SDS

Serum-free, cell-depleted 24h conditioned media of cell lines were submitted for APO, MV and EXO isolation using differential centrifugation/ultracentrifugation and gravity driven size filtration. EVs were stained with Annexin V APC and anti-CD9 FITC. APOs and MVs were analyzed directly, whereas EXOs were detected upon binding to latex beads. All fluorescent events promptly disappeared upon addition of SDS. The figure shows representative data for the lysis of THP-1 cell-derived EVs.

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