ELSEVIER
Nanomedicine: Nanotechnology, Biology, and Medicine
xx (2016) xxx–xxx
nanomedicine
Nanotechnology, Biology, and Medicine
nanomedjournal.com

Size-dependent cellular uptake of exosomes

Federica Caponnetto[a,b], Ivana Manini[b], Miran Skrap[c], Timea Palmai-Pallag[a], Carla Di Loreto[b], Antonio Paolo Beltrami[b], Daniela Cesselli[b], Enrico Ferrari[a,*]

[a]School of Life Sciences, University of Lincoln, Lincoln, UK
[b]Department of Medical and Biological Sciences, University of Udine, Udine, Italy
[c]Neurosurgery Unit, Department of Neuroscience, Santa Maria della Misericordia University Hospital, Udine, Italy

Received 26 August 2016; accepted 6 December 2016

Abstract

The ability of exosomes to elicit specific cellular responses suggests that they may be increasingly used as therapeutics. Their vesicular nature makes them suitable as potential nanocarriers for drugs or nucleic acids delivery. Here we address the question whether the method of preparation of enriched exosomal fractions can affect their uptake by cells and their ability to trigger a response. We compared ultracentrifugation and polymer-based precipitation methods on supernatants of glioma-associated stem cells isolated from a high-grade glioma patient. We determined particle size distributions after purification and their correlation with uptake, proliferation and migration in glioblastoma cell cultures. Our findings indicate that polymer-based precipitation leads to smaller particle size distributions, faster uptake by target cells and increased cellular motility. The different effect that isolation method-dependent populations of particles have on cell motility suggests their size distribution could also profoundly affect exosome therapeutic potential.

Key words: Exosomes; Glioma; GASC; Cellular uptake; AFM; Flow cytometry

Exosomes are extracellular membrane vesicles found in many body fluids and released by a variety of cell types.[1,2] It has been shown that exosomes play an active role in cell-to-cell communication[3–6] and, for example, contribute to determine the tumor micro-environment with consequences for proliferation, invasion and metastasis.[7] Much attention has been focused on the molecular characterization of exosome content, primarily nucleic acids and proteins, and on the specific markers exposed on the lipid vesicles that determine specific interactions with target cells.[8] However, it has been suggested that physical properties of the particles may also affect the way exosomes mediate intercellular communication.[9] In fact, in the case of engineered nanoparticles, it has been shown that their size may affect the uptake efficiency and kinetics, the internalization mechanism and also the subcellular distribution.[10] Whether the size of natural vesicles might be also an essential factor that determines how easily they can diffuse in a tissue and how effectively cells can take them up is still unknown and represents the overarching question behind this study.

Exosome size is reported to vary from 30 to 100 nm[1,2] and, as a consequence of the small nature of particles, an accurate estimation tends to be elusive. Several techniques are available to determine the size of nanoparticles that have been applied to characterize exosomes. These include transmission electron microscopy (TEM), atomic force microscopy (AFM), nanoparticle tracking analysis (NTA) and dynamic light scattering (DLS) among the most used.[11–17] Unfortunately, none of these methods on its own have the ability to estimate the size distribution accurately and at the same time provide high-throughput. Microscopy techniques, TEM and AFM, provide a direct measurement of individual particles size but generally on samples with small numbers of particles, whereas light scattering techniques, NTA and DLS, quickly provide size distributions based on much larger sample size, however, the size estimation is not direct and prone to artifacts. There is also a degree of diversity in the methods used for the enrichment of the exosomal

Abbreviations: GASC, GLIOMA-ASSOCIATED STEM CELLS; DiD, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine perchlorate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Funding: Timea Palmai-Pallag was funded by the BBSRC grant BB/K019597/1.

Conflict of interest: No competing interests are present.

*Corresponding author at: School of Life Sciences, University of Lincoln, LN6 7TS Lincoln, UK.
E-mail address: eferrari@lincoln.ac.uk (E. Ferrari).

http://dx.doi.org/10.1016/j.nano.2016.12.009
1549-9634/

fractions, mostly based on precipitation by ultracentrifugation or polymers.[13] Different methods can lead to preparations with different properties, including a different size distribution and possibly a proportion of extracellular vesicles other than exosomes.[9,18]

A comprehensive and accurate understanding of the actual size distribution of exosome preparations might not be a priority for functional studies or biomarker discovery, but it is of vital importance when moving toward the use of exosomes as therapeutics.[19] In fact it is known that the physical properties of lipid vesicles, including size, can have a dramatic effect on cells ability to internalize them.[20] Therefore, a better knowledge of the effect of extraction method on particles size distribution and cellular uptake would be beneficial in this context and could also contribute to the general understanding of the mechanism of exosome uptake in intercellular communication.[21] Apart from their potential use as therapeutics, exosomes could be used as predictors of disease status.[22] For example, glioma-associated stem cells (GASC), a population of stem cells isolated from the glioma microenvironment and endowed with a significant prognostic potential, exert their tumor supporting activity through the release of exosomes.[23] Also in this context, besides the already well-studied molecular profiles, a better understanding of the physical properties of exosomes might be informative and useful to predict the clinical outcome of diseases.[24]

Here we purified exosomal fractions from GASC supernatants, derived from a high-grade glioma patient stem cells, using two well established and widely used methods: ultracentrifugation (UC) and ExoQuick (EQ) precipitation.[9,13,18,25] We then accurately characterized the resulting particles size distribution using primarily AFM and NTA. We exposed A172 glioblastoma cell cultures to set amounts of fluorescently labeled exosomes and evaluated the cellular uptake by fluorescence microscopy and flow cytometry. We finally verified whether differential uptake of exosome preparations could affect glioma cell motility.

Methods

Cell cultures

Human glioma samples were collected at the Department of Neuroscience, Santa Maria della Misericordia University Hospital in Udine, after a written informed consent from the patient was obtained, in accordance with the declaration of Helsinki, and with approval by the independent ethics committee of the hospital. Glioma associated stem cells (GASC) and A172 human glioblastoma cells were cultured as described in[26,27] and detailed in the Supplementary methods.

Exosome purification, size and density measurements

A172 and GASC supernatants were processed using either ExoQuick (EQ) according to manufacturer's protocol or ultracentrifugation (UC) precipitation as previously described.[13] Size and density measurements were performed using AFM, NTA and DLS. Purification, characterization methods and protein assays for density estimation are detailed in the Supplementary methods section.

Exosome uptake assays

Purified exosomes were labeled by DiD (Life Technology) and re-precipitated to remove the excess of fluorophore. Uptake was analyzed by confocal or epi-fluorescence microscopy as detailed in Supplementary methods. Flow cytometry was performed on a FACSverse flow cytometer (BD Biosciences) on cells detached from substrates with TrypLE Express solution (Life Technologies), washed and resuspended in 200 μl PBS. After reaching a count of 1×10^4 cells, the data were gated on the basis of forward and side scattering and the mean fluorescence intensity (MFI) was calculated as the ratio between total DiD fluorescence over the number of cells showing fluorescence for both DiD and Hoechst.

Scratch assay

On a 24-well plate 1×10^5 cells were seeded and brought to high cellular confluence then scratched using a 200 μl tip. Phase contrast images of the scratches were acquired every 6 h, until their complete closure, by a Nikon Eclipse TS100 microscope (Nikon) and a 10× objective. The velocity (μm/h) was calculated by measuring the distance covered into the scratch by the front of migration in several points. Images were processed and distances calculated using ImageJ (National Institutes of Health).

Statistical analysis of size, uptake and migration measurements is detailed in the Supplementary methods.

Results

Effect of the extraction method on the size distribution of exosomal fractions

EQ and UC isolated exosomes from three GASC cultures supernatants derived from the same patient were adsorbed on mica coverslips and imaged using AFM to estimate the size distribution of the particles in the exosome fractions. Figure 1, *A* and *B* are representative 5 × 5 μm images of EQ and UC purified exosomes respectively, obtained from the same supernatant. Although both preparations produced individually separated particles with a near-spherical morphology, the EQ method generated particles that appeared to be smaller. This was confirmed by a quantitative analysis on a large number of particles from 10 images acquired from the two preparations. Supplementary Figure 1, *A* shows the size distributions in which the size of individual particles was determined from the maximum height of each particle. The analysis of the distributions confirmed that EQ purification leads to smaller particles than UC and the resulting peak size average over the three replicates, reported in Table 1, confirmed the significant difference in particle size of extracellular vesicles obtained with the two methodologies. To investigate whether this observation was limited to the case of GASC only or could be a more general effect due to the nature of the extraction method, we repeated the experiment using the same exact approach on exosomes extracted from three supernatants of A172 glioblastoma cell cultures. Also in this case the results confirmed that exosomes isolated by EQ were significantly smaller than those obtained by