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Characterisation of exosomes derived from human cells by nanoparticle tracking analysis and scanning electron microscopy

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ABSTRACT

Exosomes from three different cell types (HEK 293T, ECFC, MSC) were characterised by scanning electron microscopy (SEM), dynamic light scattering (DLS) and nanoparticle tracking analysis (NTA). The diameter was around 110 nm for the three cell types. The stability of exosomes was examined during storage at −20 °C, 4 °C, and 37 °C. The size of the exosomes decreased at 4 °C and 37 °C, indicating a structural change or degradation. Multiple freezing to −20 °C and thawing did not affect the exosome size. Multiple ultracentrifugation also did not change the exosome size.

1. Introduction

Initially exosomes were described as vesicles that were released by rat reticulocytes as a consequence of the fusion of multivesicular endosomes with the plasma membrane [1,2]. More than a decade later, exosomes were isolated from B lymphocytes, and it was demonstrated that as antigen-presenting vesicles, these exosomes can induce T-cell responses [3]. Mainly within the last 5 years, the research on exosomes started to burst and it became evident that exosomes are released from an increasing variety of different cells types. In addition to a number of proteins being involved in intercellular cell signalling, they have been found to transport micro as well as messenger RNAs that can be incorporated and translated in target cells [4]. Acting as multi-signalling batteries they can modulate the immune response, and tumour cells were shown to use such mechanisms to escape from the host immune system [5,6]. As they contain cell-specific signatures analyses of exosomes may be used for diagnostic purposes, *e.g.* in melanoma [7] or in ovarian cancer diagnostics [8]. Additionally, positive impacts of exosomes on tissue regeneration have been observed [9]. Thus, in addition to being very interesting intercellular signal mediators whose function will be elaborated within the next few years, exosomes appear as promising new tools for the clinical diagnostics and maybe for novel treatment strategies [10,11].

Up to now, exosomes have mainly been studied with biological techniques, which is often very challenging due to their comparatively small size of 50–100 nm [12]. Scanning electron microscopy (SEM) and atomic force microscopy was reported by Sharma et al. [13]. Here, we applied two well-established colloid-chemical techniques [14], *i.e.* SEM and dynamic light scattering (DLS), and one comparatively new technique, *i.e.* nanoparticle tracking analysis, to characterise the size of exosomes derived from different human cell types. The exosome size was followed during storage at −20 °C (including multiple thawing), 4 °C, and 37 °C, and also during ultracentrifugation. These are all established techniques to handle exosomes in biology, and it is of prime importance to assess the integrity of exosomes under these conditions.

2. Materials and methods

2.1. Cells and culture conditions

Cells of the human embryonic kidney cell line HEK 293T were cultured in Dulbecco's Modified Eagle Medium (DMEM; PAA

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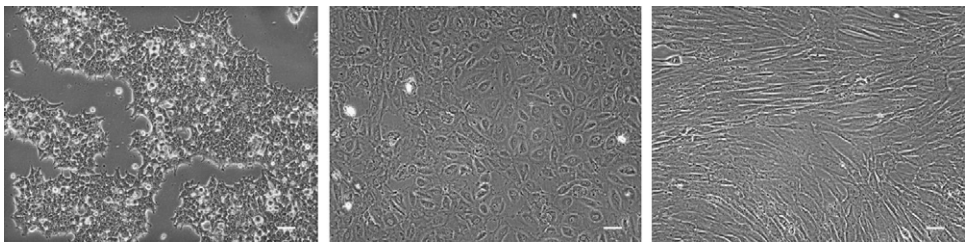

Fig. 1. Light-microscopic images of the exosome-producing cells: HEK 293T (left), human ECFC (center) and MSC (right) (scale bar: 100 μm).

Laboratories, Paschingen, Austria) supplemented with 10% fetal calf serum (FCS; Biochrom AG, Berlin, Germany) and 1% penicillin, streptomycin, glutamine (Gibco, Invitrogen GmbH, Frankfurt, Germany). Primary human mesenchymal stem/stroma cells (MSCs) which had initially been raised from umbilical cord tissue were cultivated in alpha-Minimum Essential Medium (alpha-MEM, PromoCell GmbH, Heidelberg, Germany) supplemented with 10% FCS of a selected, pretested batch (PAN-Biotech GmbH, Passau, Germany). Primary endothelial colony forming cells (ECFC) which had initially been raised from umbilical cord blood were cultured in EGM2 Medium (Lonza Cologne GmbH, Cologne, Germany). One day after cell culture, the supernatants were harvested for the isolation of exosomes, the former media were removed, the cells were washed once with phosphate-buffered saline (PBS) and the supernatant of fresh media was added that had been depleted from serum exosomes by ultracentrifugation at 110,000 × *g* for 2 h.

2.2. Exosome isolation and purification

Exosomes were isolated from cell culture supernatants of HEK 293T cells, ECFCs and MSCs. To remove cells, conditioned media were centrifuged for 5 min at 900 × *g*, and to remove remaining debris, another centrifugation step was performed for 1 h at 10,000 × *g*. To remove all particles bigger than 200 nm, the supernatants were filtered through 0.2 μm pore filters. To concentrate the exosomes, the filtrate was passed through a Vivacell 100 Filter (Sartorius AG, Goettingen, Germany) during 30 min centrifugation at 4 °C and 400 × *g*. 10 mL of the concentrated supernatant were passed through a Sepharose CL-2B column (1.5 cm × 45 cm; GE Healthcare, Munich, Germany) and 1 mL fractions were eluted. Following the estimation of the protein contents by a Bradford Ultra protein assay (Expedeon Ltd., Harston, UK), the protein-containing fractions belonging to the main peak were pooled and centrifuged for 2 h at 110,000 × *g* at 4 °C. The obtained precipitates were resuspended in 150 μL PBS and used for further colloid-chemical analyses.

2.3. Analytical methods

For scanning electron microscopy (ESEM Quanta 400 instrument; FEI), the exosomes were fixed with 3.7% glutaraldehyde (Sigma–Aldrich GmbH, Taufkirchen, Germany) in PBS for 15 min. After washing twice with PBS, the fixed exosomes were dehydrated with an ascending sequence of ethanol (40%, 60%, 80%, 96–98%). After evaporation of ethanol, the samples were left to dry at room temperature for 24 h on a glass substrate, and then analysed by SEM after gold–palladium sputtering.

Dynamic light scattering and zeta potential determinations were performed with a Zetasizer nanoseries instrument (Malvern Nano-Zetasizer, λ = 532 nm laser wavelength). The exosome size data refers to the scattering intensity distribution (*z*-average). For particle size determination, nanoparticle tracking analysis (NTA) was performed with a NanoSight LM10 instrument equipped with the NTA 2.0 analytical software. All experiments were carried out at 1:1000 dilution, leading to particle concentrations around 6×10^7 mL^{-1}. Each experiment was carried out in triplicate. The 50% median value (D50) is given in all cases, and the standard deviation is given for all data. The particle size distribution in a typical experiment was D10 183 nm, D50 258 nm, D70 277 nm, and D90 381 nm. For dynamic light scattering and nanoparticle tracking analysis, the exosomes which were initially dispersed in PBS after the isolation were diluted 1:1000 with pure water. The salt concentrations after dilution were 137 μM NaCl, 2.7 μM KCl, 8.1 μM Na_2HPO_4, and 1.76 μM KH_2PO_4 at a pH of 7.4. The total ion strength was therefore about 166 μM.

Multiple centrifugation steps to assess the stability of the exosomes were performed three times for 2 h each and once for 16 h at 110,000 × *g* at 4 °C. Following each centrifugation step, the precipitates were resuspended in 200 μL PBS. For analytical purposes 50 μL of each obtained sample were retained. Multiple deep-freezing of the exosomes was accomplished by freezing of the exosome dispersion (10 mL) at −20 °C for 24 h, followed by thawing under gentle shaking at room temperature for about 5 min. For the NTA experiment, 1 mL of the samples was measured for about 15 min, returned into the storage vessel and deep-frozen again.

3. Results and discussion

Three different exosome-producing human cell types were used (Fig. 1). We used exosomes isolated from a human embryonic kidney cell line (HEK 293T), from human umbilical cord blood-derived endothelial cells, so-called ECFC [15], and from human umbilical cord derived MSCs.

Fig. 2 shows the results of SEM. All exosomes had a spherical shape with a diameter of about 30–50 nm. Agglomeration occurred due to the drying process before the SEM analysis. The size in dynamic light scattering was 212 nm (PDI = 0.626) for HEK exosomes, 226 nm (PDI = 0.481) for ECFC exosomes and 208 nm (PDI = 0.605) for MSC exosomes. Note that the high polydispersity index (PDI) indicates a multimodal particle size distribution. In dynamic light scattering, the exosomes showed a strongly negative zeta potential of −54 ± 6 mV for HEK exosomes, −49 ± 13 mV for ECFC exosomes and −52 ± 4 mV for MSC exosomes due to the negatively charged phospholipid membrane.

The size of the exosomes from nanoparticle tracking analysis (Fig. 3) was around 120 nm for HEK exosomes, 110 nm for ECFC exosomes, and 110 nm for MSC exosomes. The difference to the results from SEM (30–50 nm) compared to nanoparticle tracking analysis (around 110 nm) is due to the fact that the latter one monitors the hydrodynamic diameter of the exosomes in solution, and also that larger particles contribute more strongly to the light scattering than the smaller particles which leads to a shift compared to the D50 value from NTA.

Even though the human cells which we used in this study are morphologically very heterogeneous (Fig. 1), their exosomes had comparable sizes and shape (Fig. 2). Recently, it has been shown that components of the evolutionary conserved ESCRT complexes deform the membrane of late endosomes in a defined manner to