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Research Paper

Effect of exosome isolation methods on physicochemical properties of exosomes and clearance of exosomes from the blood circulation

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ABSTRACT

Exosomes, which are expected to be delivery systems for biomolecules such as nucleic acids, are collected by several methods. However, the effect of exosome isolation methods on the characteristics of exosomes as drug carriers, such as recovery efficiency after sterile filtration and pharmacokinetics, has not been investigated despite the importance of these characteristics for the development of exosome-based delivery systems. In the present study, exosomes collected from murine melanoma B16-BL6 cells by several methods were compared with respect to dispersibility, recovery rate after filtering, and clearance from the blood circulation in mice. The exosomes were collected by three ultracentrifugation-based methods: simple ultracentrifugation/pelleting (pelleting method), ultracentrifugation with an iodixanol cushion (cushion method), and ultracentrifugation on an iodixanol density gradient (gradient method). The isolation methods had little effect on the particle number of exosomes. In contrast, transmission electron microscopy observation and size distribution measurement using tunable resistive pulse sensing indicated that the exosomes of the gradient method were more dispersed than the others. The exosomes were labeled with *Gaussia* luciferase and intravenously injected into mice. Clearance of injected exosomes from the blood circulation did not significantly change with isolation methods. When the exosomes were filtered using a 0.2-μm filter, the recovery rate was 82% for the exosomes of the gradient method, whereas it was less than 50% for the others. These results indicate that the exosome isolation method markedly affects the dispersibility and filtration efficiency of the exosomes.

1. Introduction

Exosomes are extracellular vesicles with a diameter of 30–150 nm that contain biomolecules including RNAs and proteins [1–3]. Exosomes are secreted from most types of cells and play roles in intercellular communication. Exosomes derived from specific cells have therapeutic potential. For example, exosomes derived from mesenchymal stem cells exert protective effects on myocardial ischemia/reperfusion injury [4]. Thus, exosomes themselves are expected to be used in disease treatments. They are also expected to be used as a biocompatible and efficient delivery carrier given that they are endogenous intercellular delivery carriers that transfer biomolecules including RNAs and proteins [5–7]. Exosomes as well as exosome-mimetic vesicles may be used to deliver therapeutic molecules such as small interfering RNAs, microRNAs (miRNAs), and small-molecule drugs including curcumin, doxorubicin, and porphyrin to target cells [8–12].

Exosomes are usually collected from cell culture medium in such applications as delivery carrier and therapeutic treatment. There are several methods for isolating exosomes from the medium such as ultracentrifugation-based, solvent precipitation, column chromatography, and immunoaffinity methods [13]. The most commonly used method of isolation is the ultracentrifugation-based method. This method has a low cost and a low risk of contamination with separation reagent, and allows collection of large numbers of exosomes [4,11]. Several ultracentrifugation-based methods have been developed for isolating exosomes. Another frequently used method is based on solvent precipitation [14]. This method is performed by incubation of samples with solvent reagent followed by centrifugation at a relatively low speed, and it does not require ultracentrifugation. The isolation methods influence the composition of miRNA and protein of exosomes [15,16] as well as exosome morphology [17]. However, little is known about the effect of isolation methods on the yield and physicochemical properties of exosomes, such as size, zeta potential, and dispersibility, despite the fact that these parameters affect the characteristics of exosomes as a delivery carrier and may change the therapeutic effect and delivery efficiency of

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exosome-based treatments. Although a few studies have compared the yield and physicochemical properties of exosomes obtained by different methods [18,19], whether or not differences in physicochemical properties influence the characteristics of exosomes as drug carriers has not been investigated.

In the present study, we selected the solvent precipitation method and three ultracentrifugation-based methods, namely simple ultracentrifugation/pelleting (pelleting method), ultracentrifugation with a cushion at the bottom of the tubes (cushion method), and density gradient ultracentrifugation (gradient method) as exosome isolation methods and investigated the effects of these isolation methods on the characteristics of the collected exosomes. We used B16-BL6 murine melanoma cells to obtain exosomes because these cells produce large numbers of exosomes and B16 cell line-derived exosomes have frequently been characterized [2,20]. The fusion protein of *Gaussia* luciferase (gLuc) and lactadherin (gLuc-LA) was then used as a probe to evaluate the effects of the methods on the clearance of exosomes from the blood circulation [21,22].

2. Materials and methods

2.1. Cell culture and transfection

B16-BL6 murine melanoma cells were obtained and cultured as described previously [21]. Plasmid DNA encoding gLuc-LA was constructed in the previous study [21]. To obtain gLuc-LA-labeled exosomes, plasmid DNA encoding gLuc-LA was transfected to B16-BL6 cells with polyethyleneimine (PEI) "max" (Polysciences, Warrington, PA, USA). Briefly, 128 μL PEI "max" solutions of 0.323 mg/mL, pH 8.0, and 16 μg plasmid DNA were individually diluted to 500 μL with 150 mM NaCl. They were mixed and incubated at 15 min to make a complex. The solution was added to the cells. The medium was changed to Opti-MEM 4 h after transfection, and cells were incubated for additional 24 h.

2.2. Exosome collection

Cells were seeded in 150-mm dishes at a cell number of 5.0×10^6 cells per dish and cultured for 24 h. Then, the cell culture medium was removed and cells were washed thrice with phosphate-buffered saline (PBS). Next, 30 mL of Opti-MEM was added to the dish and the cells were incubated for additional 24 h. The culture medium was processed before ultracentrifugation by differential centrifugation at 300*g* for 10 min, 2000*g* for 20 min, and 10,000*g* for 30 min to remove cell debris and large vesicles. In addition, the medium was filtered with a 0.2-μm syringe filter. All the ultracentrifugation steps described below were performed using a Himac CP80WX ultracentrifuge (Hitachi Koki, Tokyo, Japan).

In the pelleting method, the processed cell culture medium was spun at 100,000*g* in an angle rotor (P50AT2, Hitachi Koki) for 1 h to obtain pellets containing exosomes. The pellet was then resuspended in PBS and spun again at 100,000*g* in the angle rotor for 1 h. The exosomes obtained were resuspended in PBS.

In the cushion method, 9 mL of the processed cell culture medium was overlaid onto 40% OptiPrep (Axis-Shield PoC, Oslo, Norway) in 0.25 M sucrose/10 mM Tris–HCl, pH 7.5 and was spun at 100,000*g* in a swinging-bucket rotor (P40ST, Hitachi Koki) for 1 h. An aliquot from the border zone of the medium and the OptiPrep was collected and resuspended in PBS and was spun at 100,000*g* in the angle rotor for 1 h to obtain pellets containing exosomes. The exosomes were resuspended in PBS.

In the gradient method, 40%, 20%, 10%, and 5% OptiPrep solutions were prepared by dilution of a stock solution of OptiPrep with 0.25 M sucrose/10 mM Tris–HCl, pH 7.5. The gradient was formed by sequentially layering 2.5 mL of each solution in descending order of density. The processed cell culture medium was concentrated to 1 mL using ultrafiltration (100 K), layered on the top of the gradient layer, and spun at 100,000*g* in the swinging bucket rotor for 18 h. Then, 1 mL of each fraction was collected sequentially from the top and numbered by collection order. Thus, the density of the collected fraction increased with fraction number. It was expected that exosomes would be recovered in fraction 9, based on the density of this fraction. After confirmation by a preliminary experiment that exosome marker proteins were detected mostly in this fraction, the fraction was used without additional treatment. In preliminary experiments, we confirmed that iodixanol remaining in the sample did not influence the assays, including filtration and pharmacokinetic analysis (unpublished results).

In solvent precipitation, exosomes were collected from the processed cell culture medium using Exoquick-TC (System Biosciences, Mountain View, CA, USA) according to the manufacturer's instruction.

2.3. Western blotting

Exosome samples (0.05 μg protein) and cell lysate of B16BL6 (1 μg protein) were reduced by addition of 100 mM dithiothreitol and heat treatment at 95 °C for 3 min. The samples were then electrophoresed on 10% sodium dodecyl sulfate–polyacrylamide gel. Proteins were transferred from the gel to polyvinylidene fluoride membrane. The membrane was soaked with Blocking One (Nacalai Tesque, Kyoto, Japan) for 30 min. Then the membrane was soaked with antibody solutions to detect Alix and heat shock protein (HSP) 70, both of which are exosome marker proteins. To detect Alix, monoclonal mouse anti-Alix antibody (BD Biosciences, San Jose, CA, USA) was used at 1:1000 dilution, followed by anti-mouse IgG1 horseradish peroxidase-conjugated antibody (Life Technologies, Grand Island, NY, USA) at 1:1000 dilution. To detect HSP70, monoclonal mouse anti-HSP70 antibody (Cell Signaling Technology, Danvers, MA, USA) was used at 1:2000 dilution, followed by goat anti-rabbit IgG antibody conjugated with horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1:5000 dilution. To detect CD63, polyclonal rabbit anti-CD63 antibody (Santa Cruz Biotechnology) was used at 1:200 dilution, followed by goat anti-rabbit IgG antibody conjugated with horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1:5000 dilution. To detect calnexin, polyclonal rabbit anti-calnexin antibody (Santa Cruz Biotechnology) was used at 1:200 dilution, followed by goat anti-rabbit IgG antibody conjugated with horseradish peroxidase (Santa Cruz Biotechnology) at 1:5000 dilution. The membrane was soaked with Immobilon Western Chemiluminescent HRP Substrate (Merck Millipore, Billerica, MA, USA), and chemiluminescence was observed with a LAS3000 instrument (Fujifilm, Tokyo, Japan).

2.4. Transmission electron microscopy (TEM)

Exosomes were fixed in 4% paraformaldehyde and layered on a carbon/Formvar film-coated TEM grid (Okenshoji Co., Ltd., Tokyo, Japan) for 20 min. The grids were treated with 1% glutaraldehyde and washed eight times with dH_2O for 2 min. They were then stained with 1% uranyl acetate for 10 min. Observation was performed with a transmission electron microscope (Hitachi H-7650, Hitachi High-Technologies Corporation, Tokyo, Japan). To measure Feret's diameter of the particles, TEM images were analyzed using ImageJ software (Rasband, W.S., U.S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997–2014).