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Visualization and in vivo tracking of the exosomes of murine melanoma B16-BL6 cells in mice after intravenous injection

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ABSTRACT

The development of exosomes as delivery vehicles requires understanding how and where exogenously administered exosomes are distributed in vivo. In the present study, we designed a fusion protein consisting of *Gaussia* luciferase and a truncated lactadherin, gLuc-lactadherin, and constructed a plasmid expressing the fusion protein. B16-BL6 murine melanoma cells were transfected with the plasmid, and exosomes released from the cells were collected by ultracentrifugation. Strong luciferase activity was detected in the fraction containing exosomes, indicating their efficient labeling with gLuc-lactadherin. Then, the labeled B16-BL6 exosomes were intravenously injected into mice, and their tissue distribution was evaluated. Pharmacokinetic analysis of the exosome blood concentration–time profile revealed that B16-BL6 exosomes disappeared very quickly from the blood circulation with a half-life of approximately 2 min. Little luciferase activity was detected in the serum at 4 h after exosome injection, suggesting rapid clearance of B16-BL6 exosomes in vivo. Moreover, sequential in vivo imaging revealed that the B16-BL6 exosome-derived signals distributed first to the liver and then to the lungs. These results indicate that gLuc-lactadherin labeling is useful for tracing exosomes in vivo and that B16-BL6 exosomes are rapidly cleared from the blood circulation after systemic administration.

1. Introduction

Cells secrete various types of nanosized membrane vesicles, including exosomes, which are secretory vesicles with diameters of 50–120 nm (Johnstone, 1992). It has been reported that exosomes contain the microRNA (miRNA) of exosome-producing cells and that exosomes are taken up by both the cells that produce them and other cells opened the possibility of intercellular exchange of miRNA (Valadi et al., 2007). Since these findings, the roles of exosomes in intercellular communication have become important topics of scientific interest. It has been demonstrated that exosomes transduce intercellular information through miRNA, mRNA, proteins, and lipids, all of which have been found in exosomes (Pan and Johnstone, 1983; Subra et al., 2010; Théry et al., 2009). Exosomes play important roles in numerous biological events, such as inflammation and tumor metastasis, by mediating intercellular communication (Peinado et al., 2011; Théry et al., 2009).

As exosome-mediated intercellular communication occurs through the transduction of materials in exosomes, such as miRNA, exosomes can be safe and efficient delivery vehicles for bioactive materials. The development of exosomes as delivery vehicles requires understanding and quantitatively analyzing their pharmacokinetics, i.e., their in vivo fate after administration. Exosomes were previously used for the delivery of small interfering RNA (siRNA) and curcumin, an anti-inflammatory agent, to target cells (Alvarez-Erviti et al., 2011; Sun et al., 2010; Zhuang et al., 2011). Alvarez-Erviti et al. developed an exosome-based carrier system for the delivery of siRNA to the brain (Alvarez-Erviti et al., 2011). They detected fluorescently labeled siRNA in the brain cells of mice after the intravenous injection of genetically modified exosomes collected from dendritic cells. Sun et al. administered exosomes labeled with a fluorescent probe and found that the labeled exosomes distributed primarily to the kidneys, liver, spleen, and lungs (Sun et al., 2010). Although these preceding studies revealed some aspects of exosomes in vivo, no pharmacokinetic data are available for exogenously administered exosomes. One possible reason why the pharmacokinetics of exosomes has not been completely investigated thus far is the lack of sensitive methods to quantitatively evaluate exosome levels in vivo.

In our previous studies, we demonstrated that the tissue distribution of exogenously administered cancer cells could be quantitatively evaluated by genetically labeling the cells with firefly luciferase, a chemiluminescence-emitting enzyme (Hyoudou et al., 2004; Takahashi et al., 2005). Based on these results, we hypothesized that labeling exosomes with any reporter protein that emits chemiluminescence will permit quantitative evaluation of the

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tissue distribution of exosomes in vivo. With this aim, we selected two proteins: *Gaussia* luciferase (gLuc) and lactadherin. The former is a reporter protein that emits chemiluminescence when its substrate, coelenterazine, is present, and the latter is a membrane-associated protein mainly found in exosomes. Lactadherin is composed N-terminal secretion signal peptide domain, EGF-like domain, and C-terminal lectin type C1/C2 (C1C2) domains. It was reported that the N-terminal secretion signal and C1C2 domains of lactadherin are required for lactadherin to be transported to exosomal compartments and to be retained on the membranes of exosomes, respectively (Delcayre et al., 2005). On the other hand, the EGF-like domain contains arginine–glycine–aspartic acid (RGD)-motif, and it was reported that lactadherin-bound vesicles are recognized by phagocytes via the RGD-motif of lactadherin (Dasgupta et al., 2009). Therefore, we designed a fusion protein consisting of gLuc, N-terminal secretion signal of lactadherin and C1C2 domains of lactadherin (gLuc-lactadherin). We collected exosomes produced from B16-BL6 murine melanoma cells after transfection with gLuc-lactadherin-expressing plasmid vector. Then, we performed the visualization and in vivo tracking of the labeled exosomes after intravenous injection into mice.

2. Materials and methods

2.1. pDNA

pDNA encoding gLuc (pCMV-gLuc) was constructed as described previously (Takiguchi et al., 2011). The cDNA of murine lactadherin was purchased from Open Biosystems (Thermo Fisher Scientific K. K., Tokyo, Japan). The sequence of gLuc was fused to the C1C2 domain of mouse lactadherin by PCR using the following primers: gLuc (forward) 5′-GGATAGATCTCAGCATGCAGGTCTCCC-GTGTGCTGGCCGCGCTGTGCGGCATGCTACTCTGCGCCTCTGGCCTC-TTCGCCGGTAAGCCCACCGAGAACAA-3′, (reverse) 5′-TTCCATG-CCCAGCTGTGTAGAACAACCGTCACCACCGGCCCCCTTGATCT-3′; lactadherin C1C2 (forward) 5′-TGTTCTACACAGCTGGGCATGGA-3′, (reverse) 5′-CTACGCTAGCCTGAGCATTAACAGCCCAGCAG-3′. The chimeric sequence of gLuc-lactadherin was subcloned into the *Bam*HI/*Xba*I site of the pcDNA 3.1 vector (Invitrogen, Carsbad, CA, USA) to construct pCMV-gLuc-lactadherin.

2.2. Cell culture and transfection

The B16-BL6 murine melanoma cell line was obtained from the Cancer Chemotherapy Center of the Japanese Foundation for Cancer Research. B16-BL6 cells were cultured in Dulbecco's modified Eagle's minimum essential medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin/L-glutamine (PSG) at 37 °C and 5% CO_2. B16-BL6 cells plated on culture plates were transfected with pDNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. In brief, 1 μg of pDNA was mixed with 3 μg of Lipofectamine 2000 and dissolved in OPTI-MEM I (Invitrogen) at a final concentration of 2 μg of pDNA/ml. The resulting complex was added to the cells and incubated for 4 h. Then, the remaining complex was removed by replacing the medium, after which it was incubated for an additional 20 h.

2.3. Collection of exosomes, gLuc protein, and gLuc-lactadherin protein free from exosomes

One day before collecting the exosomes, the culture medium of B16-BL6 cells was replaced with DMEM supplemented with PSG and FBS. To avoid contamination of the FBS-derived exosomes, FBS was spun at 100,000 × *g* for 2 h to remove exosomes before use. The culture supernatants were collected after 24 h of incubation, and the exosomes in the supernatants were purified by ultracentrifugation according to the method of Théry et al. (2006). In brief, the culture supernatants were cleared of cell debris and large vesicles by sequential centrifugation at 300 × *g* for 10 min, 1000 × *g* for 20 min, and 10,000 × *g* for 30 min, followed by filtration using 0.2-μm syringe filters (filtrate). Then, the cleared sample was spun at 100,000 × *g* for 1 h to pellet the exosomes, and supernatant was collected as the ultracentrifuge supernatant sample (UC supernatant). The collected exosomes were washed twice with PBS. The recovery of exosomes was estimated by measuring the protein concentration using the Bradford assay. To collect exosome-free gLuc protein or gLuc-lactadherin protein, UC supernatant was collected from the cells that were transfected with pCMV-gLuc or pCMV-gLuc-lactadherin. Each sample was mixed with a sea pansy luciferase assay system (Picagene Dual; Toyo Ink, Tokyo, Japan), and the chemiluminescence was measured with a luminometer (Lumat LB 9507; EG&G Bethhold, Bad Wildbad, Germany).

2.4. Dynamic light scattering, zeta potential analysis and electron microscopic observation of exosomes

The exosome suspension (approximately 0.5 mg protein/ml) was added to an equal volume of 4% paraformaldehyde, and the mixture was applied to a carbon/Formvar film-coated TEM grid (Alliance Biosystems, Osaka, Japan) and incubated for 20 min at room temperature. After washing with PBS, the sample was fixed by incubation with 1% glutaraldehyde for 5 min, washed with PBS, and then incubated with 1% uranyl acetate for 5 min. The sample was observed under a transmission electron microscope (Hitachi H-7650, Hitachi High-Technologies Corporation, Tokyo, Japan). The particle size and zeta potential of the collected exosomes were determined using a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) at 20 °C.

2.5. Gaussia luciferase zymography

Each sample was electrophoresed under nonreducing conditions on 10% SDS-polyacrylamide gels. Gels were washed twice with 2.5% Triton X-100 for 30 min and once with PBS for 30 min. The gels were then soaked in Piccagene Dual, and chemiluminescence was observed using an LAS3000 instrument (Fujifilm, Tokyo, Japan).

2.6. Sephacryl S-400 gel filtration

Sephacryl S400 (GE Healthcare, Little Chalfont, Buckinghamshire, England) was added to micro Bio-spin chromatography columns (Bio-Rad) that were subsequently equilibrated with PBS. Exosomes, free gLuc, or free gLuc-lactadherin was applied to the column and eluted with PBS by gravity flow. Collected fractions were weighed to determine the volume, and the gLuc activity in each fraction was determined by the aforementioned procedure.

2.7. Animals

Five-week-old male C57BL/6 and BALB/c mice were purchased from Japan SLC, Inc. (Shizuoka, Japan). C57BL/6 mice, which are syngeneic to B16-BL6 cells, were used for all the experiments except for in vivo imaging study, in which white BALB/c mice were used to sensitively detect the chemiluminescence. All protocols for the animal experiments were approved by the Animal Experimentation Committee of Graduate School of Pharmaceutical Science of Kyoto University.