Evaluation of the pharmacokinetic and pharmaceutical characteristics of exosomes for the development of exosome-based drug delivery carrier.

(エキソソームを利用したデリバリーキャリアの開発を目的とした体内動態および製剤学的特性の評価)

２０１８

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Preface

Intercellular communication is essential in multicellular organisms. Cells exchange information through various pathways. Cells communicate with neighbor cells by direct contact between cells such as gap junction. On the other hand, cells secrete molecules like hormones or cytokines that send signal to distant cells. Besides these molecules, cells also secrete extracellular vesicles that transfer cargoes to their recipient cells[1]. Extracellular vesicles are roughly classified into three types based on their origin: apoptotic bodies, microvesicles, and exosomes [2]. Among these extracellular vesicles, exosomes have gained much attraction.

Exosomes are small membrane vesicles with a diameter of 30–120 nm that are secreted from various types of cells [3,4]. Since the discovery that exosomes act as intercellular communication tools by transferring their cargoes including proteins and nucleic acids to the recipient cells, the roles of exosomes in physiological events such as tumor metastasis and immune response have been vigorously investigated [5–7]. In addition, the possibility of the development of exosome-based drug delivery systems (DDS) has been demonstrated by several studies in which exosomes were used to deliver proteins and nucleic acids to specific types of target cells [8,9]. To exploit exosomes as drug delivery carriers, it is important to understand the factors affecting the pharmacokinetics of exosome, such as types of exosome-producing cells, and the role of surface protein on their pharmacokinetics. In addition, preservation method is an important issue to be concerned for the development of exosome-based drug delivery carriers. However, the information about these factors is limited.

Therefore, in this thesis, I investigated the pharmacokinetics of exosome from five different types of murine cell lines, and elucidated the role of exosome surface protein on their pharmacokinetics by developing method to label inner space of exosomes. In addition, I also developed a preservation method of exosomes utilizing lyophilization.

Chapter 1

Evaluation of cell type-specific and common characteristics of exosomes derived from mouse cell lines

1-1. Introduction

Exosomes are small membrane vesicles secreted from various types of cells, which transfer their cargoes, proteins and nucleic acids, to the recipient cells. Several studies demonstrate that exosomes can be exploited as drug delivery vehicles, which can deliver proteins and nucleic acids to specific types of target cells [2,8,9].

The yield of exosomes and the physicochemical properties that affect their pharmacokinetics, such as particle size and surface charge, may vary with the type of exosome-producing cell. Because these factors are expected to greatly influence the therapeutic efficacy of exosomes, it is necessary to select appropriate types of exosome-producing cells for the development of exosome-based DDS. Moreover, the exosome yield is an important factor for the development of exosome-based DDS. However, little information is available about how the yield, physicochemical properties, and pharmacokinetics of exosomes depend on the cell type.

In this chapter, five different types of murine cell lines, which represent whole body of mouse, were selected as model exosome-producing cells: B16BL6 melanoma cells, C2C12 myoblast cells, NIH3T3 fibroblasts cells, MAEC aortic endothelial cells, and RAW264.7 macrophage-like cells. B16BL6 cell line was selected as a control because my laboratory reported the pharmacokinetics of B16BL6-derived exosomes[10]. I also selected other 4 types of normal, not tumor, cell lines, because these cells were easily transfected and produced gLuc-LA-modified exosomes. I collected exosomes from these types of cells and evaluated the exosome yield by measuring protein amount and particle number. I then investigated the particle size and zeta potential of these exosomes. To evaluate the pharmacokinetics of exosomes after an intravenous injection into mice, a fusion protein of *Gaussia* luciferase (gLuc) and lactadherin (LA), gLuc-LA, was used to label the exosomes with gLuc [10]. The time course of the serum exosome concentration was examined by measuring the gLuc activity after intravenous injection of gLuc-LA-labeled exosomes, and the biodistribution of the labeled exosomes was visualized using *in vivo* imaging.

1-2. Material and Methods

1-2-1. Cell culture

B16BL6 cells were obtained from the Cancer Chemotherapy Center of the Japanese Foundation for Cancer Research. C2C12, NIH3T3, and RAW264.7 cells were purchased from the American Type Culture Collection. MAEC cells were a gift from Professor Ichiro Saito (Department of Pathology, Tsurumi University School of Dental Medicine, Yokohama, Japan). B16BL6 cells, C2C12 cells, and NIH3T3 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). RAW264.7 cells were cultured in Roswell Park Memorial Institute medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% FBS and PSG. MAEC cells were cultured in medium 199 (Gibco, Grand Island, NY, USA) supplement with 10% FBS and PSG. Cells were cultured at 37°C in humidified air containing 5% CO_2.

1-2-2. Collection of exosomes

Culture media used for exosome collection were prepared by ultracentrifugation at 100,000 × g for 2 h to remove FBS-derived exosomes. To reach approximately 80% confluency after 24 h incubation, cells were seeded into 15-cm dishes at the following numbers: 8×10^6 cells for B16BL6 and C2C12, 7×10^6 cells for NIH3T3, 5×10^6 cells for MAEC, and 2×10^7 cells for RAW264.7. Twenty-four hours after cell seeding, the medium was replaced with exosome-depleted medium and incubation was resumed for another 24 h. At the time of harvesting exosomes, the confluency of cells was almost 100% for all the cases examined. Exosomes in the supernatant were purified using a previously described procedure [10]. In brief, cell debris and large vesicles were cleared from the supernatant by sequential centrifugation and filtration using a 0.2 μm filter. Subsequently, the supernatant was subjected to ultracentrifugation at 100,000 × g for 1 h to sediment the exosomes. The exosomes were washed twice with phosphate-buffered saline (PBS). The amount of collected exosomes was estimated by measuring the protein concentration

using the Quick Start Bradford protein assay (BioRad, Hercules, CA, USA), according to the manufacturer's instructions, and by measuring the particle number using a qNano instrument (Izon Science Ltd., Christchruch, New Zealand). Exosomes labeled with gLuc-LA were collected as previously described [11]. In brief, exosome-producing cells were transfected with gLuc-LA-expressing plasmid vectors using polyethylenimine "Max" (Polysciences, Warrington, PA, USA) and were incubated for 24 h. Exosomes in the culture supernatant were collected as described above. Exosomes labeled with gLuc-LA were mixed with a sea pansy luciferase assay system (Picagene Dual; Toyo Ink, Tokyo, Japan), and their chemiluminescence was measured with a luminometer (Lumat LB 9507; EG&G Bethhold, Bad Wildbad, Germany) to estimate gLuc activity. For immunofluorescent experiment, exosomes were labelled with PKH26 dye (Sigma-Aldrich) as previously described [10]. All the collected exosomes were aliquoted to avoid multiple freeze-thaw cycles and were stored at -80ºC until use. Storage of exosomes at -80ºC had negligible effect on their physicochemical properties.

1-2-3. Western blotting

Cell lysates were prepared using a freeze–thaw procedure followed by centrifugation to remove the cell debris. Western blotting was performed as previously described [11,12]. In brief, reduced exosomes and cell lysate samples (1.5 μg protein for Alix, HSP70, and calnexin and 5 μg protein for CD81) were loaded onto a 10% sodium dodecyl sulphate-polyacrylamide gel, were subjected to electrophoresis, and were then transferred to a polyvinylidene fluoride transfer membrane. The membrane was incubated with Alix-specific antibody (1:20,000 dilution; BD Biosciences, San Jose, CA, USA), HSP70-specific antibody (1:1000 dilution; Cell Signaling Technology, Danvers, MA, USA), CD81-specific antibody (1:200 dilution; Santa Cruz Biotechnology, CA, USA), or calnexin-specific antibody (1:1000 dilution; Santa Cruz) overnight at 4°C. The membranes were then incubated with horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG antibody (1:2000 dilution; Thermo Fisher, Waltham, MA, USA) or goat anti-rabbit IgG antibody (1:5000 dilution; Santa Cruz Biotechnology) for 1 h at room temperature.

The membrane was reacted with Immobilon Western Chemiluminescent HRP Substrate (Merck Millipore, Billerica, MA, USA), and chemiluminescence was detected using an LAS-3000 instrument (FUJIFILM, Tokyo, Japan).

1-2-4. Transmission electron microscopy

Exosome samples were mixed with an equal volume of 4% paraformaldehyde in PBS. The mixture was applied to a carbon Formvar film-coated transmission electron microscopy (TEM) grid (Alliance Biosystems, Osaka, Japan) and was incubated for 20 min at room temperature. After washing with PBS, the samples were fixed with 1% glutaraldehyde for 5 min. After washing with distilled water, the grid was stained with 1% uranyl acetate for 2 min, and the samples were observed using TEM (Hitachi High-Technologies Corporation, Tokyo, Japan)

1-2-5. Particle size distribution and zeta potential of exosomes

A qNano instrument was used to measure the particle size distribution of the exosomes. A Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) was used to determine the zeta potential of the exosomes.

1-2-6. Animals

Five-week-old male BALB/c mice were purchased from Japan SLC, Inc. (Shizuoka, Japan). All protocols for the animal experiments were approved by the Animal Experimentation Committee of the Graduate School of Pharmaceutical Science of Kyoto University.

1-2-7. Pharmacokinetic studies

Exosomes labeled with gLuc-LA were intravenously injected into mice via the tail vein at a dose of 5 μg/200 μl/shot. Blood samples were collected at indicated time points. Serum was obtained by centrifuging clotted whole blood at 8000 × g for 20 min at 4°C. These serum samples were diluted with PBS, and their chemiluminescence was then measured as described above. The

amount of exosomes in each sample was normalized to that of the injected dose based on the gLuc activity and was expressed as the percent of the injected dose/ml (% ID/ml). The area under the curve (AUC) and the mean residence time were calculated for each animal by integration to 4 h. The clearance (CL) was calculated by dividing the injected dose by the AUC. The half-life ($t_{1/2\alpha}$) was calculated as previously described [11].

1-2-8. Chemiluminescence imaging of exosomes *in vivo*

Mice received intravenous injections of gLuc-LA-labeled exosomes at a dose of 5 μg/200 μl/shot. Five minutes after the administration, chemiluminescent images were acquired using an IVIS Spectrum *in vivo* imaging system (Caliper Life Science, Hopkinton, MA, USA). Immediately before imaging, 100 μg of coelenterazine (Regis Technologies, Morton Grove, IL, USA), a substrate for gLuc, was injected into the tail vein of each mouse.

1-2-9. Immunofluorescent staining of macrophages

Exosomes labelled with PKH26 dye were injected into mice via the tail vein at a dose of 5 μg/200 μl/shot. Five minutes after injection, mice were sacrificed, and the liver was harvested. The harvested organs were frozen at -80ºC, and the frozen sections were prepared by using a freezing microtome (Leica CM3050 S; Leica Biosystems, Germany). The sections were stained with Alexa Fluor488-labelled anti-mouse F4/80 antibody (Biolegend, San Diego, CA, USA) as previously described [11].

1-3. Results

1-3-1. Exosomes were collected from five different cell types

Exosomes collected from five different cell types were positive for the exosome marker proteins Alix, HSP70, and CD81, although there were differences in the amount of these marker proteins among the exosomes (Fig. 1a). In particular, exosomal expression of CD81 was highly variable among the different cell types. Exosome samples were negative for calnexin, an

endoplasmic reticulum marker, suggesting that the collected exosome samples were not contaminated with cell debris. Figure 1b shows the TEM images of the exosomes. Globular vesicles of approximately 100 nm in diameter were observed in all the exosome samples.

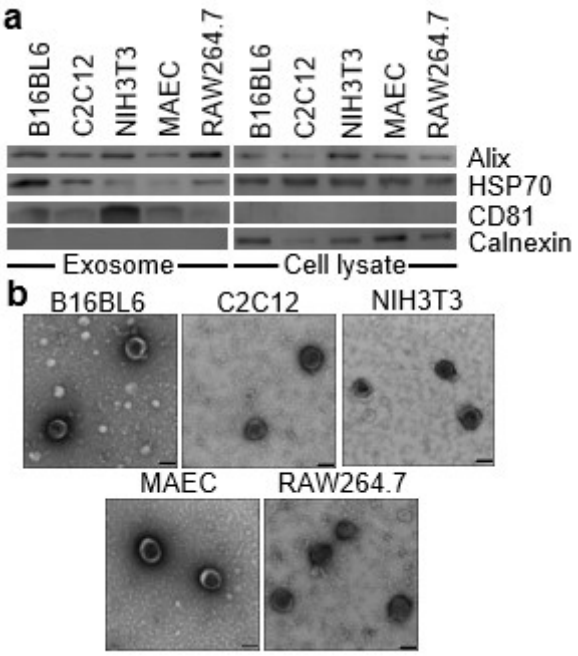

Figure 1. Collection of exosomes from five different cell types. (a) Western blotting analysis of the Alix, HSP70, CD81, and calnexin present in the exosomes and cell lysates derived from B16BL6, C2C12, NIH3T3, MAEC, and RAW264.7 cells. (b) Transmission electron microscopy images of exosomes derived from B16BL6, C2C12, NIH3T3, MAEC, and RAW264.7 cells. Scale bar = 100 nm.

1-3-2. Yield of exosomes was dependent on cell type

Figure 2 shows the amount of protein and the number of exosome particles collected from the supernatant of each culture dish after an incubation

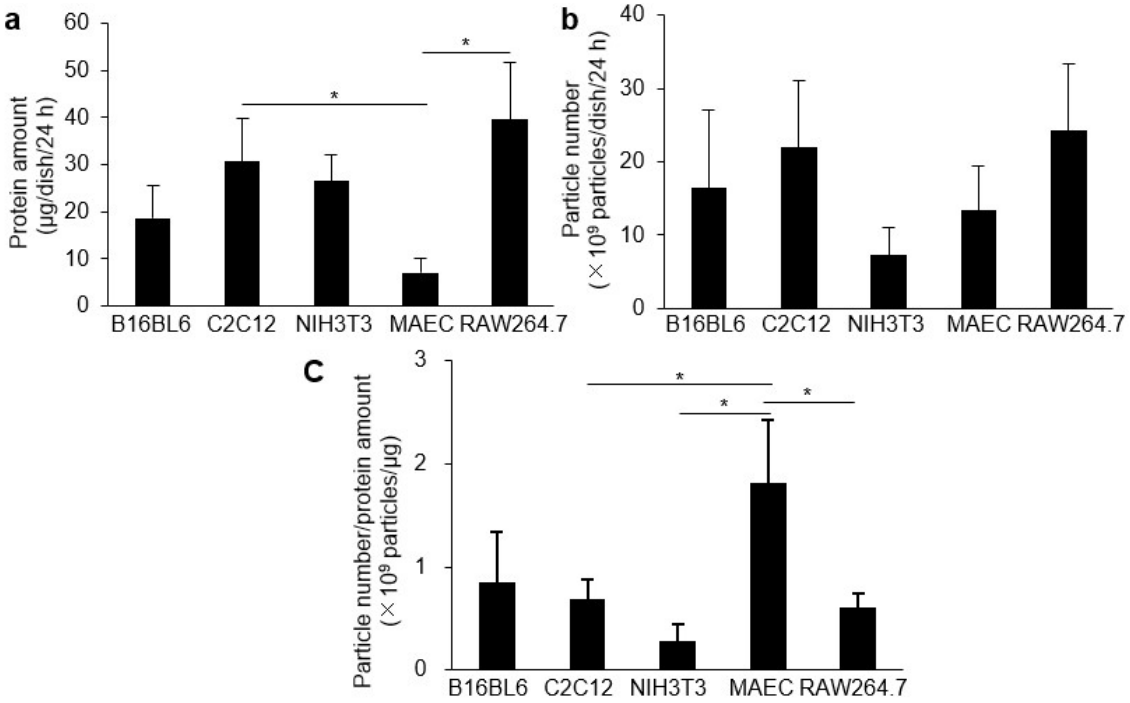

Figure 2. Amount of exosomes secreted from different cell types. (a) Amount of protein present in exosomes estimated using the Bradford assay. (b) Numbers of exosome particle estimated using a qNano instrument. (c) The ratio of exosome particles per μg protein. These results are expressed as the mean ± standard deviation (n = 3). *$p < 0.05$.

period of 24 h. Both parameters showed that C2C12 and RAW264.7 cells produced more exosomes than the other cell types. As for the particle number/protein amount ratio, MAEC showed the highest ratio, whereas NIH3T3 showed the lowest ratio among the cell types investigated in this study.

1-3-3. Exosomes were approximately 100 nm in diameter and possessed a negative charge

Figure 3 shows histograms of the particle size distributions of the exosomes. All the exosomes showed similar particle size distributions. Table 1 summarizes the median particle sizes and mean zeta potentials of the exosomes. All the exosomes were approximately 100 nm in diameter and possessed a negative zeta potential of approximately −40 mV.

Table 1. Particle sizes and zeta potentials of the exosomes from different cell types

Exosome-producing cells	Size (nm)	Zeta potential (mV)
B16BL6	100 ± 4	−39.2 ± 0.9
C2C12	111 ± 10	−38.9 ± 1.1
NIH3T3	106 ± 2	−35.3 ± 0.5
MAEC	102 ± 7	−41.6 ± 1.5
RAW264.7	105 ± 4	−38.8 ± 0.6

Results are expressed as the mean ± standard deviation (n = 3).

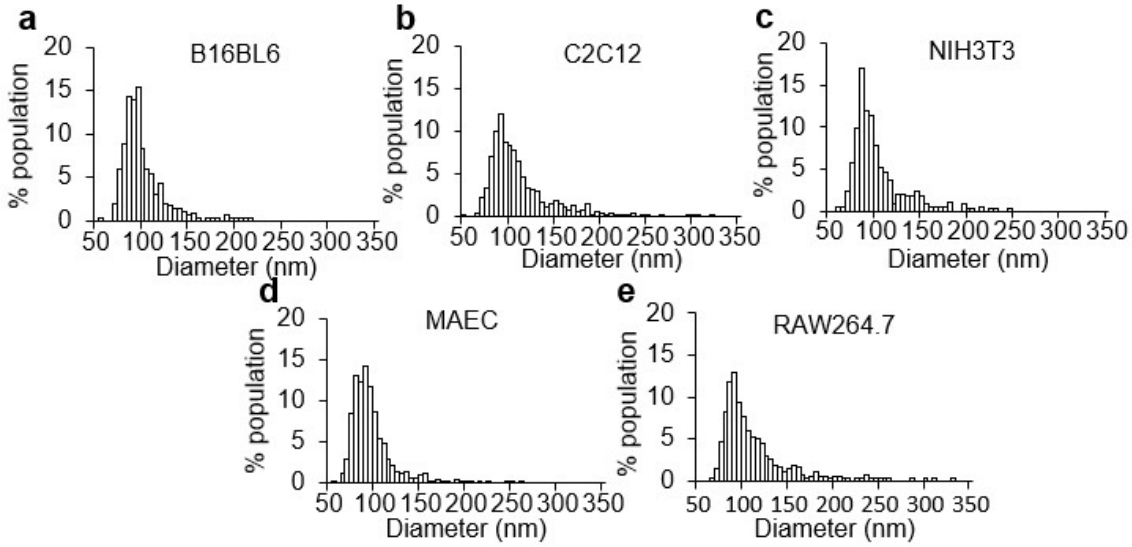

Figure 3. Exosome particle size distribution. Representative histograms of the exosome particle size distributions obtained from (a) B16BL6, (b) C2C12, (c) NIH3T3, (d) MAEC, and (e) RAW264.7 cells, determined using a qNano.

1-3-4. Exosomes were rapidly eliminated from the circulation and mainly distributed to the liver

To evaluate the pharmacokinetics of the exosomes, gLuc-LA-labeled exosomes were intravenously injected into mice. The structural and physicochemical properties of the gLuc-LA-labeled exosomes were compared with those of the unlabeled ones (data not shown). After intravenous injection into mice, serum gLuc activity immediately decreased regardless of the source of exosomes, indicating that all the exosomes were rapidly eliminated from the circulation (Fig. 4). Table 2 shows the pharmacokinetic parameters of the exosomes calculated from their serum concentration profiles. These parameters were not remarkably different among all the exosomes.

To investigate the biodistribution of the exosomes, gLuc-LA-labeled exosomes were visualized 5 min after intravenous injection using *in vivo* imaging. All the exosomes mainly distributed to the liver (Fig. 5). These results indicate that all the exosomes investigated in this study have similar biodistribution properties.

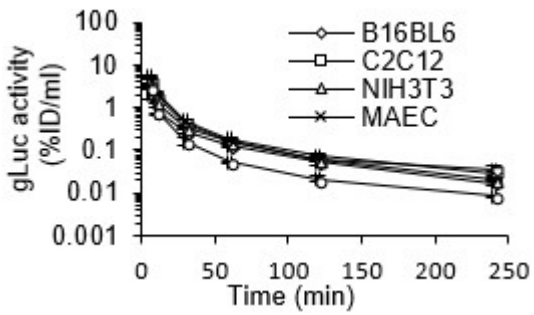

Figure 4. Time-course of serum gLuc activity after intravenous injection of gLuc-LA-labeled exosomes. Serum gLuc activity was measured after intravenously injecting gLuc-LAlabeled exosomes collected from B16BL6 (rhombus), C2C12 (square), NIH3T3 (triangle), MAEC (cross), or RAW264.7 (circle) cells. These results are expressed as the mean ± standard deviation (n = 4).

Table 2. Pharmacokinetic parameters of gLuc-LA labeled exosomes after intravenous injection

Exosome-producing cells	$t_{1/2\alpha}$ (min)	AUC (% of dose·h/ml)	MRT (h)	CL (ml/h)
B16BL6	3.81 ± 0.68	0.897 ± 0.150	0.511 ± 0.069	114 ± 19
C2C12	4.08 ± 0.52	1.54 ± 0.35	0.435 ± 0.049	67.1 ± 13.3
NIH3T3	3.92 ± 1.04	1.27 ± 0.10	0.428 ± 0.034	78.9 ± 5.9
MAEC	3.95 ± 0.73	1.87 ± 0.17	0.383 ± 0.022	53.7 ± 4.6
RAW264.7	2.77 ± 0.28	0.945 ± 0.075	0.261 ± 0.024	106 ± 8.6

These results are expressed as the mean ± standard deviation (n = 4).

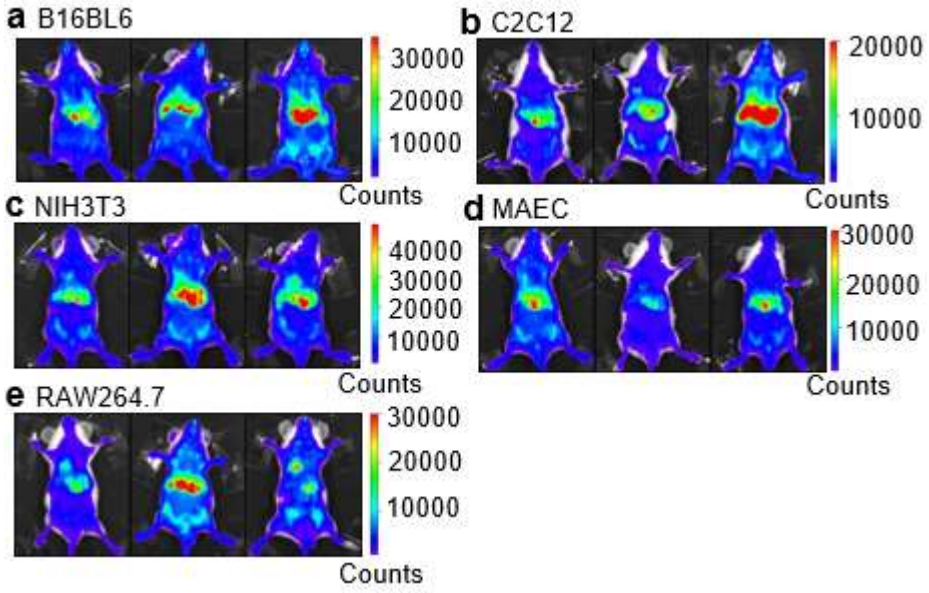

Figure 5. *In vivo* imaging of gLuc-LA-labeled exosomes. The biodistribution of gLuc-LA-labeled exosomes in mice was imaged using a bolus intravenous injection of coelenterazine 5 min after the intravenous injection of exosomes obtained from (a) B16BL6, (b) C2C12, (c) NIH3T3, (d) MAEC, or (e) RAW264.7 cells.

1-3-5. Exosomes labelled with PKH26 were taken up by macrophages

To evaluate uptake of exosomes by macrophage in the liver, PKH26-labelled exosomes were injected into mice via tail vein. Most of the red signal derived from PKH26-labelled exosomes was co-localized with F4/80+ cells stained with Alexa Fluor488 (green)-labelled antibody in the liver (Fig. 6), indicating that all types of exosomes used in this study were taken up by macrophages in the liver. In addition, previous data of my laboratory [11] showed that the clearance of B16BL6-derived exosomes was drastically delayed in macrophage-depleted mice. To investigate the role of macrophages on the clearance of other types of exosomes, all types of exosomes except B16BL6-exosomes were injected into macrophage-depleted mice. Clearance of all types of exosomes in macrophage-depleted mice

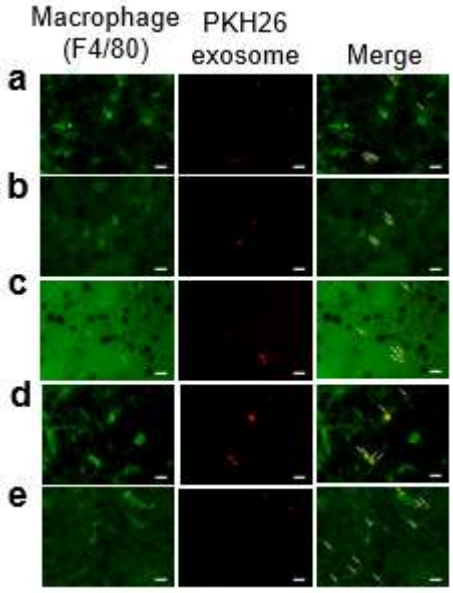

Figure 6. Macrophage uptake of exosomes labeled with PKH26. Cryostat sections of liver collected from mice receiving PKH26 (red)-labeled exosomes derived from (a) B16BL6, (b) C2C12, (c) NIH3T3, (d) MAEC, or (e) RAW264.7 cells. Macrophages were stained with F4/80-specific antibody (green). Scale bar = 10 μm.

was significantly delayed compared to that in untreated mice (Fig. 7), indicating that macrophages play a key role in the clearance of exosomes from blood circulation irrespective of the source.

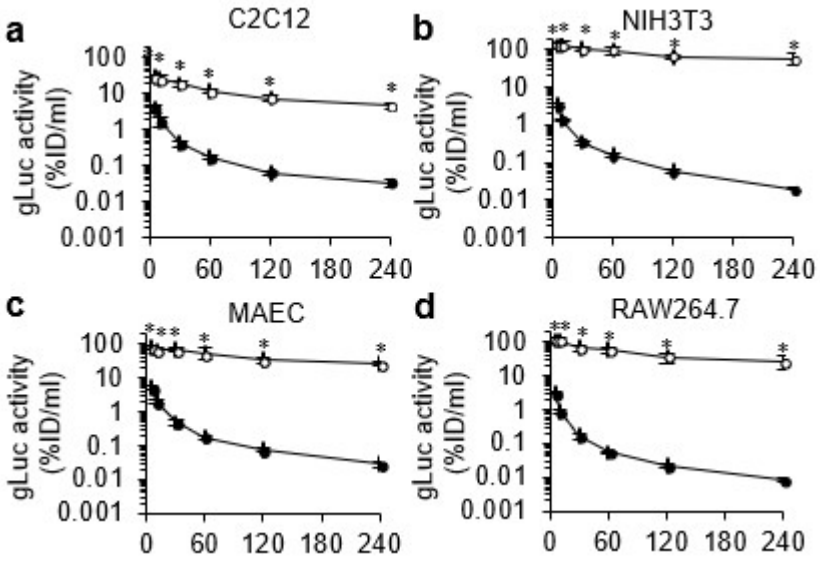

Figure 7. Clearance of exosome was delayed in macrophage-depleted mice. gLuc activity in the serum of untreated mice (closed symbols) and macrophage-depleted mice (opened symbol) was sequentially measured after intravenous injection of gLuc-LA-labelled exosomes derived from (a) C2C12, (b) NIH3T3, (c) MAEC, or (d) RAW264.7 cells. These results are expressed as the mean ± standard deviation (n = 4).

1-4. Discussion

Each cell type produced different amount of exosomes. Among the cells investigated in the present study, RAW264.7 and C2C12 produced the largest amount of exosomes. In addition, MAEC cells showed the highest particle number/protein amount ratio, whereas NIH3T3 cells showed the lowest particle number/protein amount ratio among the cell types investigated in the present study (Fig. 1a). This indicates that protein level on the exosome membrane or in the cargo is different depending on the cell type. A previous study showed that myotubes produced approximately 20 times as many exosomes as myoblasts did [13]. In previous studies on the application of exosomes as a delivery vehicle, the dose of exosomes in mice varied from 1 to 150 μg [8,14,15] and the dose of exosomes in rats varied from 50 to 250 μg [16,17]. The exosome yield of the cells investigated in this study might be enough for a dose of 1–10 μg but not for a dose of ≥150 μg because at least four 15-cm dishes are required for a single dose of 150 μg. It has been reported

that culturing cells under low pH conditions increased the yield of exosomes [18] and that an increase in cytosolic Ca^{2+} concentration stimulated exosome secretion from cells [19]. Exploitation of these methods to increase the yield of exosomes would be necessary if the exosome-based DDS required a high dose of exosomes.

An analysis of the physicochemical properties of the exosomes demonstrated that all the investigated exosomes were approximately 100 nm in diameter and had a negative zeta potential, which are consistent with preceding studies investigating the exosomes produced by the same or different cell types [20–23]. The particle size of the exosomes, a factor that will affect their pharmacokinetics, was comparable among the exosomes collected from the different cell types. In contrast, the expression levels of CD81, a tetraspanin, in the exosomes were different among the different cell types (Fig. 1a). Previous studies used fluorescein intensity measurements to demonstrate that tetraspanins, such as Tspan8, play important roles in the target cell specificity and tissue distribution of fluorescein-labeled exosomes in rats [17,24]. In addition, an *in vitro* study demonstrated that CD81 is important for the CD29-dependent autologous uptake of exosomes in mesenchymal stem cells (MSC) [25]. However, the present study revealed that the exosomes derived from the five cell types investigated here were similar in the pharmacokinetics at the whole body level. This indicates that differences in CD81 level have no significant influence on the pharmacokinetics of the exosomes in terms of serum concentration profile or biodistribution analyzed by *in-vivo* imaging.

I demonstrated that all the exosomes investigated in this study quickly disappeared from the systemic circulation and mainly distributed to the liver after intravenous injection (Fig. 4 and 5). This finding is consistent with the previous studies that investigated the pharmacokinetics of exosomes collected from various cell types [12,26,27]. In addition, the finding that exosomes distributed to the liver were mainly taken up by $F4/80^{+}$ macrophages irrespective of the type of exosome-producing cells is also in good agreement with the result of previous study of my laboratory obtained by using B16BL6-derived exosomes [11]. Moreover, this and previous study showed that the rapid clearance of exosomes from the systemic circulation was drastically delayed

in macrophage-depleted mice prepared using clodronate-containing liposome, which indicates that macrophages play a major role in the clearance of exosomes, irrespective of the type of cell producing the exosomes. It is known that macrophages efficiently take up apoptotic cells through the recognition of phosphatidylserine (PS) on their surfaces [28]. Exosomes also expose PS on their outer leaflet [29]. In addition, it has been recently reported that scavenger receptor class A family on the surface of macrophages in the liver played an important role in the uptake of extracellular vesicles [30], which is also in agreement with the findings of the current study.

In Chapter 1, I demonstrated that different cell types produced different yields of exosomes. Exosomes had comparable physicochemical and pharmacokinetics properties irrespective of types of exosome-producing cells. These findings may provide information for development of exosome-based DDS from the viewpoint of productivity of exosomes.

Chapter 2

Evaluation of the role of exosome surface proteins in the pharmacokinetics of exosome

2-1. Introduction

In the development of exosome-based drug delivery carriers, pharmacokinetics of exosomes at the whole-body level is an important issue. My laboratory previously showed that Kupffer cells in the mouse liver take up intravenously administered exosomes through the recognition of phosphatidylserine (PS) on exosomes [31]. In addition to phospholipids, it is critical to elucidate the roles of surface proteins of exosomes in the pharmacokinetics of exosome. There is little information about the role of exosome surface proteins in the pharmacokinetics of exosomes at the whole-body level, though the role of several proteins on the surface of exosomes such as tetraspanins and integrins, on the *in vivo* behavior of exosomes have been investigated. For example, it has been shown that tetraspanin Tspan8 contributes to target cell selection of exosomes [17]. Moreover, the involvement of integrins $\alpha_6\beta_4$ and $\alpha_v\beta_5$ on exosomes in cellular uptake as well as in tumor metastasis was demonstrated [32].

In Chapter 1, I tracked the whole-body distribution of exosomes by using a fusion protein termed gLuc-LA. Evaluation of the pharmacokinetics of exosome treated with proteinases would be a direct approach to estimate the contribution of surface proteins of exosomes to the pharmacokinetics of exosomes. However, gLuc-LA cannot be used for this purpose because gLuc-LA on the outer surface of exosomes would be digested by the proteinase treatment.

In this chapter, I developed a novel method to label the inner space of exosomes by using Gag protein, which is derived from Moloney murine leukemia virus (MLV). Gag localizes to the inside of plasma membrane [33] through an interaction between the polybasic region of the Gag protein and phosphatidylinositol 4,5-bisphosphate in the plasma membrane [34]. I labeled exosomes derived from B16BL6 melanoma cells through transfection of a plasmid encoding a fusion protein consisting of the Gag protein and gLuc, which I termed Gag-gLuc. Then, to confirm that Gag-gLuc exosomes could be used to examine the role of exosome surface proteins on pharmacokinetics at the whole-body level in mice, I evaluated the expression level, stability and proteinase resistance of the Gag-gLuc-labeled exosomes. Next, I evaluated the physicochemical properties of non-treated and proteinase K (ProK)-treated exosomes. Furthermore, as Gag-gLuc

localizes to the inner space of exosome, Gag-gLuc-labeled exosomes can be treated with ProK without reducing gLuc activity. Therefore, I evaluated effect of surface proteins of exosomes on their pharmacokinetics by using the labeled exosome treated with ProK.

2-2. Material and Methods

2-2-1. Plasmid DNA (pDNA)

pDNA encoding gLuc-LA was obtained as described in Chapter 1. The coding sequence of Gag, the codon sequence of which was optimized to maximize protein expression in a murine host, was synthesized by Genscript (Piscataway, NJ, USA). gLuc coding sequence was obtained as described in a previous report [35]. Coding sequence of enhanced green fluorescent protein (GFP) was prepared from the pEGFP-N1 vector (BD Biosciences Clontech, Palo Alto, CA, USA). The coding sequence of murine CD63 was purchased from Open Biosystems (Thermo Fisher Scientific, Tokyo, Japan). The chimeric sequences of CD63-gLuc, GFP-LA, Gag-GFP, gLuc-LA and Gag-gLuc were prepared by using 2-step PCR method as described in a previous report [10]. The Gag polyprotein p65 of Moloney murine leukemia virus was used in Gag-based fusion proteins. All of the sequences encoding fusion proteins were subcloned into the BamHI/Xbal site of the pcDNA3.1 vector (Thermo Fisher Scientific) to construct pCMV vectors encoding corresponding fusion proteins.

2-2-2. Cell culture

Murine melanoma B16BL6 cells were cultured as described in Chapter 1. Mouse peritoneal macrophages were collected form 5-week-old male BALB/c mice and cultured using a previously described method [31].

2-2-3. Collection of exosomes

Exosomes were collected as describe in Chapter 1. For exosomes labeled with Gag-gLuc or gLuc-LA, Gag-gLuc exosomes and gLuc-LA exosomes were lysed by lysis buffer (Pierce;

Thermo Scientific, Illinois, USA), mixed with a sea pansy luciferase assay system (Picagene Dual; Toyo Ink, Tokyo, Japan), and their chemiluminescence was measured with a luminometer (Lumat LB 9507; EG&G Bethhold, Bad Wildbad, Germany) to estimate gLuc activity. For the preparation of ProK-digested exosome, 250 μg/ml of exosome was treated with 50 μg/ml of proteinase K (Nacalai Tesque, Inc. Kyoto, Japan) for 30 min at 37 ºC. After the digestion, ProK was inhibited by incubation with 5 mM phenylmethylsulfonyl fluoride (PMSF) for 10 min at 37 ºC. Then, the samples were washed by ultracentrifugation. For uptake and distribution experiment, exosome were labeled with PKH67 (Sigma-Aldrich, St. Louis, MO, USA) and PKH26 (Sigma-Aldrich) dye, respectively, as previously described [10]. All the collected exosome samples were aliquoted to avoid multiple freeze-thaw cycles and were stored at -80 ºC until use.

2-2-4. Transmission electron microscopy

TEM observation were perform as described in Chapter 1

2-2-5. Particle size distribution and zeta potential of exosomes

Particle size distribution and zeta potential of exosomes were obtained as described in Chapter 1

2-2-6. Stability of gLuc activity and binding of Gag-gLuc and gLuc-LA to exosome in serum

Stability of exosome labeling by Gag-gLuc and gLuc-LA in the serum were evaluated as previously described [11]. In brief, samples were incubated at 37 ºC in 20% FBS in PBS solution for 4 h. Stability of gLuc enzyme activity was evaluated by measuring gLuc activity of the collect samples. Release of Gag-gLuc of gLuc-LA from exosome was evaluated by ultracentrifugation of samples at 100,000 $\times g$ 1 h at 4 ºC to pellet the exosome. Then, the amount of Gag-gLuc or gLuc-LA bound to the exosome was evaluated by estimating Gag-gLuc or gLuc-LA released from the exosome as gLuc activity in the supernatant.

2-2-7. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of exosome samples followed by Coomassie Brilliant Blue (CBB) staining

Exosome samples were reduced with dithiothreitol (Nacalai Tesque, Inc. Kyoto, Japan) at 95 ºC for 3 min. The reduced samples were loaded onto a 10% sodium dodecyl sulphate-polyacrylamide gel and were subjected to electrophoresis. The gel was stained in CBB R-250 (Wako Pure Chemical, Osaka, Japan) for 30 min at room temperature, and destained by soaking in aqueous solution containing 7.5% acetic acid and 25% ethanol for 30 min twice. The stained gel was observed using an LAS-3000 instrument (FUJIFILM, Tokyo, Japan).

2-2-8. Western blotting

Western blotting was performed as described in Chapter 1. For antibody of integrin, integrin α_6-specfic antibody (1:1,000; Cell Signaling Technology, Danvers, MA, USA) or integrin β_1-specific antibody (1:1,000; Cell Signaling Technology) were used.

2-2-9. Resistance of exosome luciferase activity to ProK treatment

Exosome samples labeled with gLuc were incubated with ProK at final concentration of 50 μg/ml at 37 ºC for 30 min. Then, 5 mM PMSF was added to the samples at 37 ºC for 10 min to inhibit ProK activity. The samples were subsequently lysed with lysis buffer and gLuc activity was measured. The gLuc activity of samples digested with ProK were calculated as the percentage of gLuc activity of untreated samples.

2-2-10. Animals

Protocols for the animal experiments were as described in Chapter 1.

2-2-11. Pharmacokinetic studies

Experiment were performed as described in Chapter 1. About dose of injection, untreated and ProK-treated Gag-gLuc labeled exosomes were intravenously injected into mice

via the tail vein at a dose of 1.4 $\times 10^{10}$ RLU/200 μl/shot (corresponding to about 8 μg/shot for untreated exosomes).

2-2-12. Macrophage uptake of exosome

Peritoneal macrophages seeded in 96-well plate (2 $\times$ 10^5 cells/well) were incubated with untreated or ProK-treated exosome labeled with PKH67 with a dose of the same fluorescence intensity (corresponding to 2.8 and 14.0 μg/ml of untreated exosome for low and high doses, respectively). Cells were then incubated at 37 ºC for 2 h, washed twice with PBS, and harvested. Mean fluorescence intensity (MFI) of the cells was measured by a flow cytometer (Gallios Flow Cytometer; Beckman Coulter, Miami, FL) to estimate the amount of exosome taken up by the cells. The data were analyzed using Kaluza software (version 1.0, Beckman Coulter)

2-2-13. Immunofluorescent staining of macrophages

Untreated or ProK-treated exosomes labeled with PKH26 were intravenously administered to mice with the same level of fluorescence intensity (corresponding to 3.4 μg/shot for untreated exosome). Ten minutes after administration, mice were reperfused with PBS and the liver was harvested. The Sections of liver were harvested and stained as described in Chapter 1. Twenty fields/section were viewed at 10× magnification under fluorescence microscopy (biozero BZ-8000; Keyence, Osaka, Japan). The number of red fluorescence signals in each field was counted using BZ-X analyzer software (Keyence).

2-2-14. Lung distribution of intravenously-administered exosomes

Untreated or ProK-treated exosomes labeled with PKH26 were intravenously administered to mice with the same level of fluorescence intensity (corresponding to 3.4 μg/shot for untreated exosome). Ten minutes after administration, mice were sacrificed, reperfused with PBS and the lungs were harvested. The harvested lungs were frozen at −80 ºC and sections were

prepared using a freezing microtome (Leica CM3050 S, Leica Biosystems, Eisfeld, Germany). Twenty fields/section were viewed at 10× magnification under fluorescence microscopy (biozero BZ-8000). The number of red fluorescence signals in each field were counted using BZ-X analyzer software.

2-2-15. Statistical analysis

Differences among data sets were statistically analyzed by either the Student's t-test for paired comparison or one-way analysis of variance (ANOVA), followed by Fisher's Protected Least Significant Difference (PLSD) test for multiple comparisons.

2-3. Results

2-3-1 Exosomes collected from cells transfected with Gag-gLuc and gLuc-LA expressing plasmid showed luciferase activity

To evaluate the efficacy of gLuc labeling by the Gag-gLuc, gLuc-LA or CD63-gLuc fusion proteins, I measured the luciferase activity of cell lysates, culture media, supernatant obtained after ultracentrifugation and exosome samples collected from cells transfected with Gag-gLuc-, gLuc-LA or CD63-gLuc-expressing pDNA. As CD63 has been often used to modify exosome membranes [36–40], CD63-gLuc was used for comparison. While the gLuc activity of cell lysates and media of the Gag-gLuc group was comparable to that of CD63-gLuc group (0.57-fold compared to CD63-gLuc), gLuc activity of Gag-gLuc exosomes was 133-fold higher than that of CD63-gLuc exosomes. However, gLuc-LA exosomes showed 35-fold higher gLuc activity than Gag-gLuc exosomes (Fig. 8).

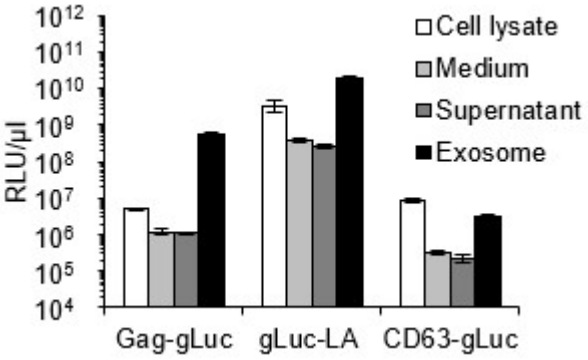

Figure 8. Exosomes can be labeled by Gag-gLuc. Luciferase activity of cell lysate (white), culture medium (light gray), supernatant after first ultracentrifugation (dark gray), and exosomes (black) collected from B16BL6 cells after transfection with Gag-gLuc, gLuc-LA, and CD63-gLuc. RLU: relative luminescence units.

2-3-2. gLuc labeling of Gag-gLuc exosome was stable in serum

gLuc activity of Gag-gLuc exosomes and gLuc-LA exosomes was stable after incubation in 20% FBS in PBS for four hours (Fig. 9a). I found that gLuc activity of Gag-gLuc exosome and gLuc-LA were 105.3% and 93.1%, respectively. Then, to evaluate the release of gLuc labeling from exosomes, Gag-gLuc exosomes and gLuc-LA exosomes were incubated in 20% FBS in PBS for four hours and were subjected to ultracentrifugation to evaluate the release of gLuc from exosomes (Fig. 9b). I found that 94.4% and 96.0% of the gLuc activity of Gag-gLuc exosome and gLuc-LA exosome, respectively, were retained, which indicates that gLuc labeling of exosomes by Gag-gLuc and gLuc-LA was stable in the serum.

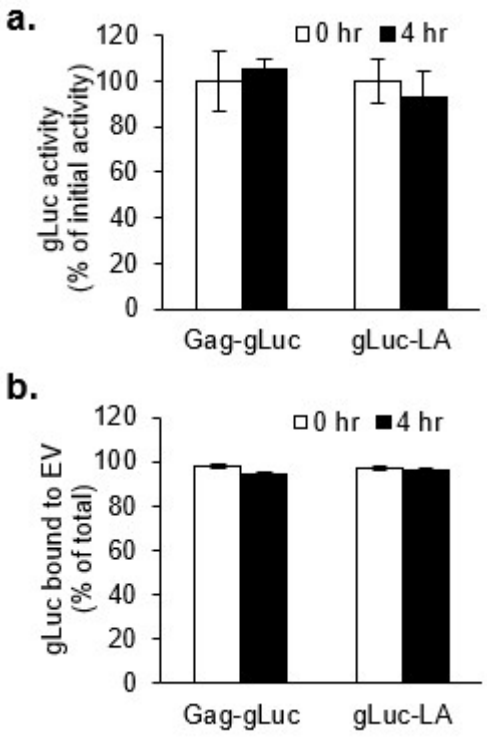

Figure 9. Exosomes labeled with Gag-gLuc and gLuc-LA were stable in serum for 4 h. Gag-gLuc exosomes and gLuc-LA exosomes were incubated in 20% FBS in PBS for 4 h. (a) Stability of gLuc activity was evaluated by measuring luciferase activity of collected samples. (b) Release of Gag-gLuc and gLuc-LA from exosomes was evaluated by measuring luciferase activity of ultracentrifugation supernatant. The data are expressed as the mean ± standard deviation (n = 3).

2-3-3. gLuc activity of Gag-gLuc exosomes and GFP signals of Gag-GFP exosomes were protected by their membrane from ProK treatment

To evaluate the resistance of gLuc activity to ProK treatment, gLuc activity of Gag-gLuc exosomes and gLuc-LA exosomes was measured after ProK treatment. gLuc activity of Gag-gLuc exosomes was not reduced by ProK treatment (99.3%), whereas gLuc activity of gLuc-LA exosome was almost completely absent (0.05%) after the digestion (Fig. 10a). I additionally confirmed that Gag-based fusion proteins were resistant to ProK treatment by observing PKH26-labeled Gag-GFP exosomes and GFP-LA exosomes under a fluorescent microscope. Figure 10b shows that the GFP signals colocalized with the red signals of PKH26-labeled exosomes in the Gag-GFP exosomes. However, for GFP-LA exosomes, the GFP signal disappeared after ProK treatment, while the red signal of PKH26-labeled exosomes was retained. These results indicate that Gag-fusion proteins are not digested by ProK treatment, suggesting that Gag-fusion proteins are located inside of exosomes.

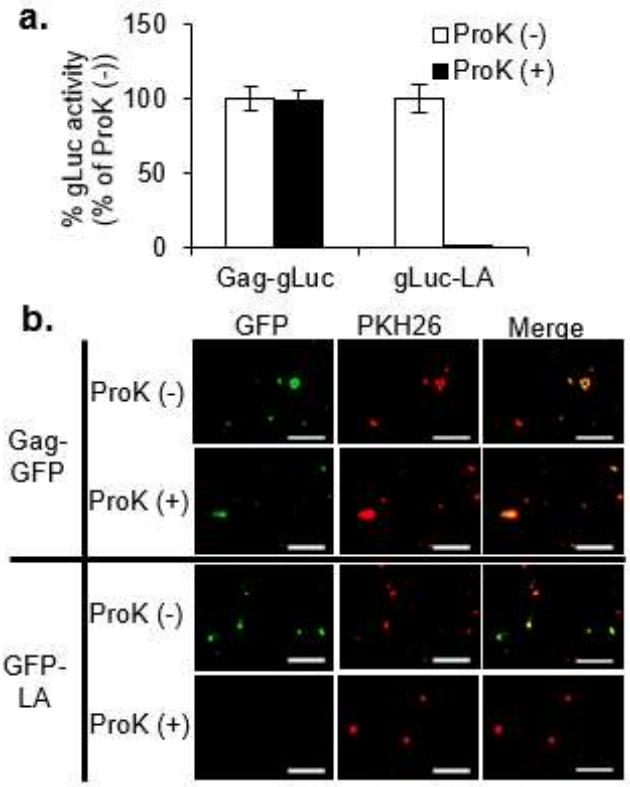

Figure 10. Gag-based fusion proteins on the interior of exosomes were resistant to proteinase K (ProK) digestion. (a) Gag-gLuc exosomes and gLuc-LA exosomes were digested by ProK, and then luciferase activity was measured. The data are expressed as the mean ± standard deviation (n = 3). (b) Gag-GFP exosomes and GFP-LA exosomes were treated with ProK and then observed by fluorescence microscopy. Scale bar = 20 μm.

2-3-4. ProK treatment digested surface proteins of exosomes without altering their physicochemical properties

To evaluate the digestion of exosomal proteins by ProK, untreated and ProK-treated exosome samples were subjected to SDS-PAGE followed by CBB staining. Figure 11a shows that some protein bands found in untreated exosomes disappeared in ProK-treated exosomes, indicating that ProK digested some exosome proteins under this condition. Figure 11b shows the western blotting analysis of exosome marker proteins in untreated and ProK-treated exosomes. Both samples were positive for Alix, an exosome marker protein localized to the interior of exosomes. I detected CD81, a tetraspanin exosome marker protein, using an antibody which reacts against helical and extracellular domains of CD81. CD81 was negative in ProK-treated exosome samples, which suggests degradation of exosome surface proteins by ProK treatment. Figure 11c shows TEM images of the untreated and ProK-treated exosomes. Globular vesicles approximately 100 nm in diameter were observed in both exosome samples. As shown in Figure 11d-f, untreated and ProK-treated exosomes showed

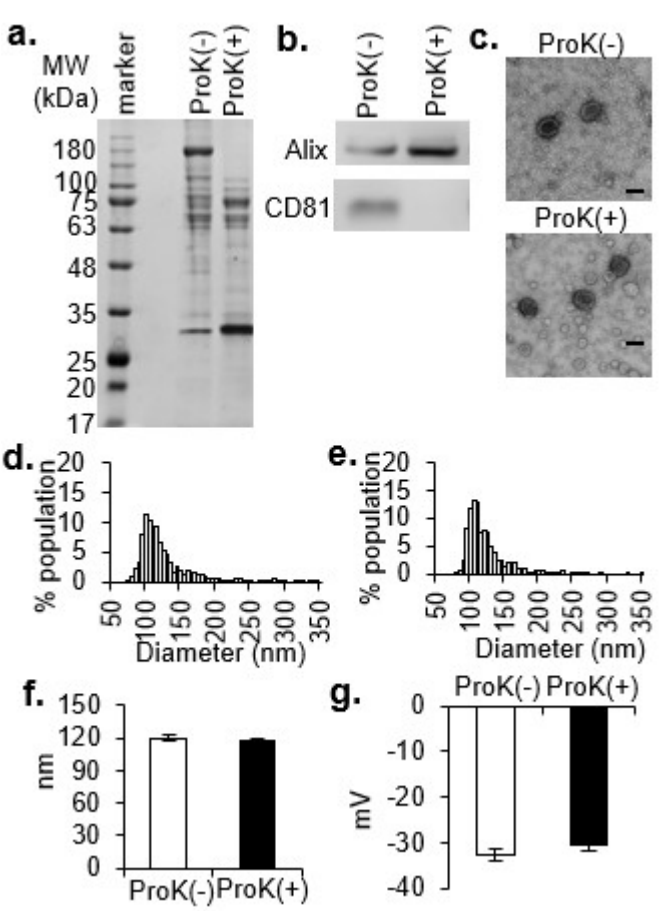

Figure 11. Effect of ProK treatment on exosome protein and physicochemical properties. (a) Coomassie Brilliant Blue (CBB) staining of untreated and ProK-treated normal exosomes. Lane 1: Marker. Lane 2: Untreated exosomes. Lane 3: ProK-treated exosomes. (b) Western blotting of Alix and CD81 in untreated (lane 1) and ProK treated normal exosomes (lane 2). (c) TEM images of untreated and ProK-treated normal exosomes. Scale bar = 100 μm. (d, e) Size distribution of untreated (d) and ProK-treated (e) normal exosomes. (f) Median size of untreated and ProK-treated normal exosomes calculated from qNano data. (g) Zeta potential of untreated and ProKtreated normal exosomes. The data are expressed as the mean ± standard deviation (n = 3).

similar size distribution and were 120 ± 3 nm and 117 ± 3 nm in diameter, respectively. Figure 11g shows that untreated and ProK-treated exosomes possessed comparable negative zeta potential, which were -32.7 ± 1.3 mV and -30.5 ± 1.3 mV, respectively. These results indicate that ProK can digest the surface proteins of exosome, but it minimally alters the physicochemical properties of exosomes.

2-3-5. ProK treatment of exosomes slightly increased their serum concentration after intravenous injection to mice

To evaluate the role of surface proteins of exosomes in pharmacokinetics, Gag-gLuc exosomes were digested with ProK, then the exosomes were intravenously injected into mice (Fig. 12). The level of initial serum concentration of Gag-gLuc exosomes treated with ProK was moderately, but significantly higher than that of the untreated group (Fig. 12a). Although the MRT and half-life did not change (Fig. 12c-d), the area under the curve (AUC) (Fig. 12b) of the ProK-treated group was significantly 1.7-fold higher than that of the untreated group.

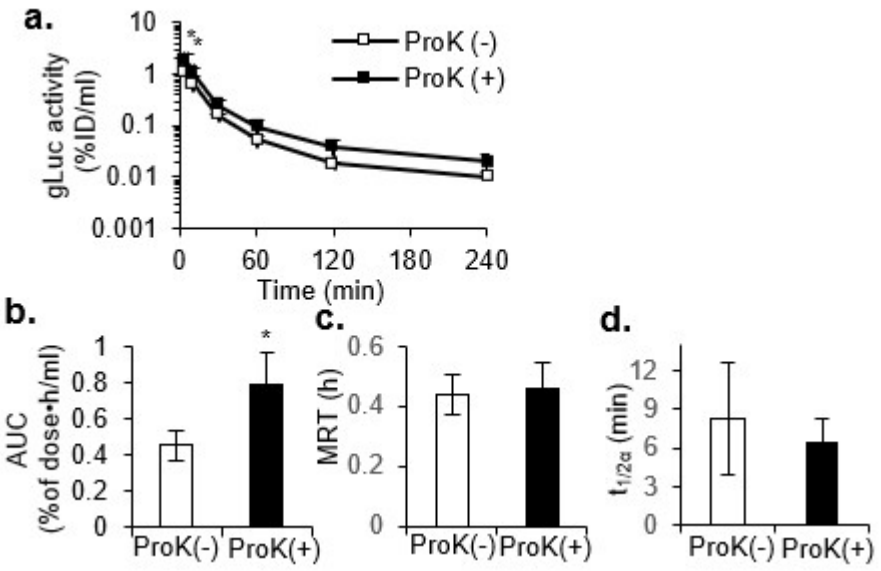

Figure 12. Time course of serum gLuc activity after intravenous injection of untreated and ProK-treated Gag-gLuc exosomes. (a) Serum gLuc activity was measured after intravenous injection of untreated and ProK-digested Gag-gLuc exosomes. These results are expressed as the mean ± standard deviation (n = 4). %ID: % of injected dose. Pharmacokinetic parameters of untreated and ProK-digested Gag-gLuc exosomes after intravenous injection, which are (b) AUC, (c) MRT, (d) $t_{1/2}$, shown as histograms. *p < 0.05 compared to untreated group. AUC values of untreated and ProK-treated exosomes were 0.46 ± 0.08 and 0.79 ± 0.18% of dose·h/mL, respectively. MRT values of untreated and ProK-treated exosomes were 0.44 ± 0.07 and 0.46 ± 0.09 h, respectively. $t_{1/2}$ values of untreated and ProK-treated exosomes were 8.30 ± 4.32 and 6.42 ± 1.19 min, respectively.

2-3-6. ProK treatment did not affect uptake of exosomes by peritoneal macrophages

Since intravenously injected exosomes are mainly taken up by macrophages in the liver and spleen [11], I evaluated the effect of surface protein digestion by ProK on the uptake of exosomes by macrophages (Fig. 13). Mean fluorescent intensity of peritoneal macrophages incubated with untreated and ProK-treated exosomes labeled with PKH67 was determined by flow cytometry. Untreated and ProK-treated exosomes showed 28.1 ± 1.1 MFI and 27.7 ± 1.1 MFI at a concentration of 2.8 μg/ml and 120.5 ± 3.7 MFI and 121.0 ± 4.5 MFI at a concentration of 14 μg/ml, respectively. There was no significant difference in the fluorescent intensity of macrophages between untreated and ProK-treated exosome groups.

To evaluate the role of surface proteins in the uptake by macrophages in the liver, co-localizations of PKH26-labelled untreated and ProK-treated exosomes with macrophage in liver section were observed. It was shown that intravenously-administered exosomes were taken up by macrophage in the liver irrespective of ProK treatment (Fig. 13b). Quantification of red

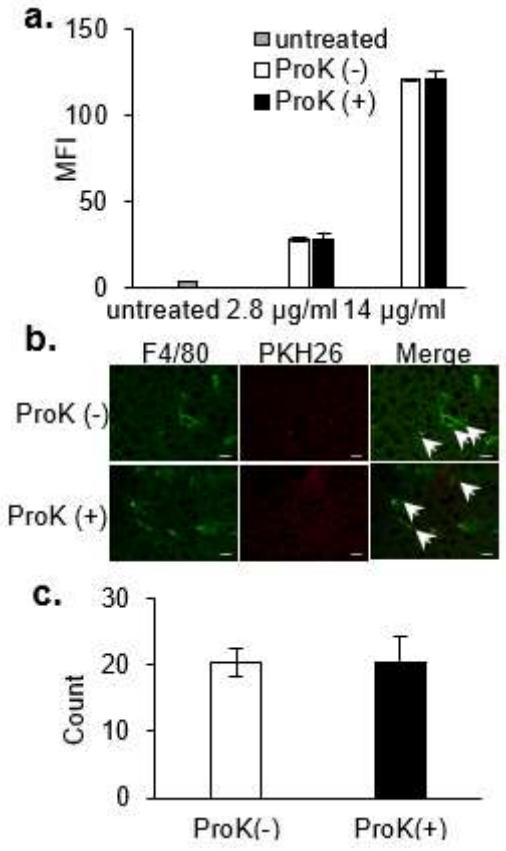

Figure 13. Uptake of untreated and ProK-treated exosomes by macrophages. (a) Peritoneal macrophages were incubated with untreated and ProK-treated PKH67-labeled exosomes, and then mean fluorescence intensity (MFI) was measured by flow cytometry. MFI values of untreated and ProK-treated exosomes were 28.1 ± 1.1 and 27.7 ± 1.1 at a concentration of 2.8 μg/mL and 120.5 ± 3.7 and 121.0 ± 4.5 at a concentration of 14 μg/mL, respectively. The data are expressed as the mean ± standard deviation (n = 4). (b) Cryostat sections of the liver collected from mice receiving PKH26-labeled exosomes (red) were stained with Alexa Fluor488-labeled anti-mouse F4/80 antibody (green). Arrowheads indicate colocalization of macrophage and exosomes. Scale bar = 10 μm. (c) Average number of red fluorescence signals was calculated from 20 fields/section. The data are expressed as the mean ± standard deviation (n = 3).

fluorescence signals showed that the amount of PKH26-labeled exosomes in the liver was comparable between untreated (20.3 ± 2.1 count) and ProK-treated (20.2 ± 4.2 count) groups. This result indicates that surface proteins are hardly involved in the uptake of exosomes by macrophages in the liver.

2-3-7. Degradation of surface proteins on exosome affected lung distribution of exosome

As it was demonstrated that $\alpha_6\beta_1$ integrin is related to lung distribution of melanoma-derived exosomes, I evaluated whether ProK treatment degraded integrin $\alpha_6\beta_1$ of exosomes. As shown in Fig. 14a, both integrin α_6 and integrin β_1 were degraded by ProK treatment. To evaluate the role of surface proteins in the distribution of exosomes to the lung, untreated and ProK-treated exosome labeled with PKH26 were injected into mice and lung sections were prepared and observed under fluorescence microscopy. Less red fluorescence was observed in the sections of lung collected from the ProK-treated exosome group (31.5 ± 4.0 counts) compared to the untreated exosome group (9.3 ± 1.0 counts) (Fig. 14a). Quantification of red fluorescence signals indicated that lung distribution of ProK-treated exosome was less than the untreated group (Fig. 14b). This result indicates that surface protein played a role in the lung distribution of exosomes.

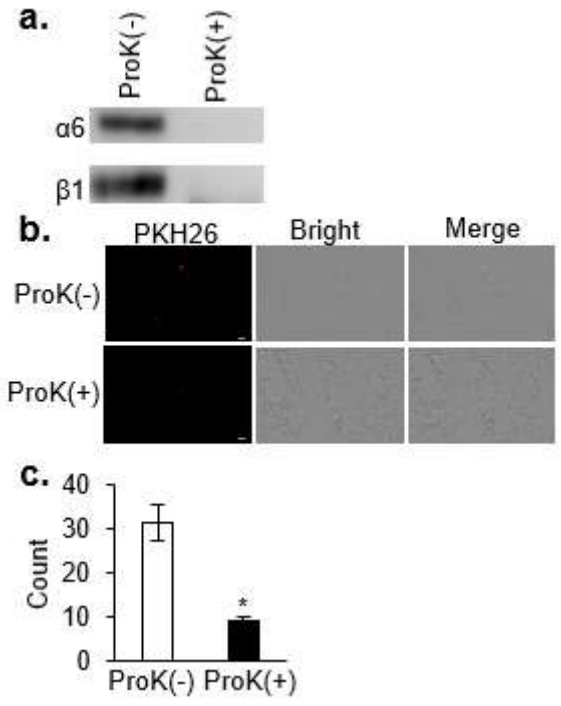

Figure 14. Lung distribution of untreated or ProK-treated PKH26-labeled exosomes. (a) Western blotting of integrin α_6 and integrin β_1 in untreated (left) and ProK-treated normal exosomes (right). (b, c) Mice were intravenously injected with exosomes. Then, 10 min after the injection, the lung samples were collected and prepared. (b) The lung samples were observed under fluorescence microscopy. (c) average number of red fluorescence signals was calculated from 20 fields/section. The data are expressed as the mean ± standard deviation from three mice. *p < 0.05 compared to untreated group.

2-4. Discussion

In the present study, I successfully labeled the inner space of exosomes by using Gag-based fusion proteins. Western blotting analysis of exosome marker proteins indicated that ProK could not digest proteins inside of the exosomes (Fig. 11). Therefore, ProK can be used for the evaluation of the role of surface proteins on exosomes. As Gag-based fusion proteins were resistant to ProK treatment, Gag-based fusion proteins appear to be located inside of exosomes (Fig. 10). Although CD63 has been widely used to label exosome membranes, Gag fusion proteins could modify exosome membranes more efficiently than CD63 because gLuc activity of Gag-gLuc exosomes was more than 100-fold higher than that of CD63-gLuc exosomes despite the fact that gLuc activity of cell lysates, which at least partly reflect the transgene expression level, was comparable between Gag-gLuc and CD63-gLuc groups. Therefore, Gag can be used to load cargoes into exosomes.

As proteinase treatment digests proteins located on the surface of exosomes, there was a possibility that properties of exosome, such as diameter and zeta potential, are affected by proteinase treatment [41]. I evaluated the total protein amount per particle of untreated and ProK-treated exosomes and found that approximately half of the total proteins in ProK-treated exosome were reduced (data not shown). Unexpectedly, although surface proteins were digested by ProK, ProK treatment had little effect on exosome physicochemical properties (Fig. 11). It was reported that the negative charge of cancer exosomes was due to the large amount of sialic acids, which are likely to be removed by ProK treatment [42]. On the other hand, as demonstrated in a previous report of my laboratory [31], phosphatidylserine, a negatively charged phospholipid, exists on the surface of exosome. As phosphatidylserine is not affected by ProK treatment, the negative zeta potential of ProK-treated exosome may be due to negatively charged lipids such as phosphatidylserine. As the physicochemical properties of delivery carrier such as size and zeta potential play important role in the pharmacokinetics of the carriers [43–45], the fact that ProK treatment minimally affected these characteristics of exosome suggests that ProK treatment can

be used for the evaluation of the roles of surface proteins on the pharmacokinetics of exosomes at the whole-body level in mice.

By using Gag-gLuc labeled exosome, I found that surface protein digestion of B16BL6-derived exosomes slightly, but significantly increased the AUC after intravenous injection of exosomes, but had little effect on other parameters. Since unchanged half-life regardless of an increase in AUC implies a decrease in distribution volume, which may be explained by the decrease in the distribution to liver and lung, I evaluated the effect of ProK treatment on the distribution to these organs. Distribution to the liver mainly occurs through uptake of exosomes by macrophages, so I evaluated uptake of exosomes by macrophages and found that removal of exosome surface proteins did not affect the cellular uptake by macrophages. Moreover, an *in vivo* experiment clearly showed that distribution to the liver and the uptake by macrophages in the liver of exosomes after intravenous administration were hardly affected by ProK treatment. In contrast, integrin $\alpha_6\beta_1$ was degraded after ProK treatment. ProK-treated exosomes showed lower lung distribution than untreated exosomes, indicating that surface proteins of exosomes are important distribution of exosomes to the lungs. It has been demonstrated that integrin $\alpha_6\beta_1$ was expressed on melanoma cells [46] and increased exosome uptake in lung and promoted the adhesion of tumor exosome within lung [32]. Therefore, surface proteins of exosomes, such as integrin $\alpha_6\beta_1$, might be related to the lung distribution of B16BL6-derived exosomes. In previous study of my laboratory, it was demonstrated that the negative charge of PS in exosome membranes is involved in the exosome uptake by macrophages [31]. The result in the current study that surface protein digestion showed little effect on exosome uptake by macrophages supports the hypothesis that PS, not protein, is the major component recognized by macrophages.

In Chapter 2, I have developed inner membrane modification method using Gag fusion protein. Using Gag fusion proteins, I revealed that some surface proteins such as integrin $\alpha_6\beta_1$ play role in their pharmacokinetics at the whole body level in mice.

Chapter 3

Development of preservation method of exosomes at room temperature by using lyophilization.

3-1. Introduction

The applications of exosomes can be expanded by the development of an appropriate preservation method. Exosomes are generally stored at -80 °C [47]; however, some reports have shown that exosomes are not stable in long-term storage under such conditions. It has been reported that the size of the exosomes decreases when stored at 4 °C or 37 °C, indicating a structural change or degradation of the exosomes [22]. In addition, a 2-year storage period led to the degradation of exosomal RNA, even at -80 °C [48]. These findings indicate that the storage of exosomes is an important issue.

Lyophilization is a technique that has been used to preserve various types of biological materials such as proteins [49], plasma [50], and living cells [51–53]. Moreover, lyophilization has been used to improve the long-term stability of nanosized drug delivery carriers, such as liposomes[54–56]. Consequently, it is possible that lyophilization may be able to improve the preservation stability of exosomes. Furthermore, lyophilized exosomes are expected to be suitable for handling if they can be stored under room temperature conditions. Currently, no data is available to suggest whether lyophilization is applicable to exosomes or not.

In this chapter, I first developed a method to lyophilize exosomes. Lyophilization removes water from frozen samples by sublimation and desorption in a vacuum [57–59]. During the freezing and drying steps, samples are under various stresses, which may damage the exosomes. To protect them from these stresses, I used trehalose, a nonreducing homodisaccharide consisting of 2 units of glucose, which is widely used as a cryoprotectant [60–62]. Next, I investigated the effect of lyophilization on the physical properties of exosomes. Finally, I evaluated the pharmacokinetics, exosomal protein and RNA content, and the activity of cargo proteins and DNA, in lyophilized exosomes and those stored at -80 °C.

3-2. Materials and Methods

3-2-1. Cell culture

Murine melanoma B16BL6 cells were cultured as described in Chapter 1.

3-2-2. Collection of exosomes from B16BL6 cells

Exosomes were collected as describe in Chapter 1. For the preparation of exosomes modified with immunostimulatory CpG DNA (CpG-exo), exosomes collected from B16BL6 cells transfected with pDNA expressing streptavidin-LA were incubated with biotinylated CpG DNA as previously described [63]. Collected exosomes were aliquoted and stored at -80 °C or subjected to lyophilization and stored at room temperature.

3-2-3. Lyophilization of exosomes

Aliquots of freshly prepared exosomes were rapidly frozen in the absence or presence of 50 mM trehalose in liquid nitrogen. Samples were lyophilized in a vacuum overnight using Eyela FDU-2100 freeze dryer (Eyela, Tokyo, Japan) and stored at room temperature for 1-4 weeks until use. Before use, lyophilized exosomes were rehydrated with water of the original volume and vortexed immediately before the analysis.

3-2-4. Determination of the polydisperse index and zeta potential

A Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) was used to determine the polydisperse index (PDI) and zeta potential of the exosomes.

3-2-5. Transmission electron microscopy

TEM observation were perform as described in Chapter 1

3-2-6. Stability of gLuc activity of gLuc-LA exosomes

gLuc activity of gLuc-LA exosomes was measured immediately following preparation from the culture medium using a luminometer (Lumat LB 9507; EG&GBethhold, Bad Wildbad, Germany). After the storage at the indicated condition for 4 weeks, gLuc activity of gLuc-LA

exosomes was measured again. The percentage of gLuc activity in exosomes after a 4-week storage period compared to that of freshly prepared exosomes was calculated.

3-2-7. Western blotting

Western blotting was performed as described in Chapter 1.

3-2-8. Animals

Protocols for the animal experiments were the same as those described in Chapter 1.

3-2-9. Pharmacokinetic studies

After storage at the indicated condition for 4 weeks, pharmacokinetic studies were performed as described in Chapter 1.

3-2-10. RNA extraction and PAGE analysis

RNA was extracted from 100 μg of exosomes stored for 1 week at -80 °C or at room temperature after lyophilization with 50 mM trehalose using Sepasol-RNA I Super G (Nacalai Tesque, Kyoto, Japan) according to the manufacturer's instructions. The RNA was then loaded onto a 9% Tris-borate-EDTA (TBE)-urea polyacrylamide gel (PAGE) and subjected to electrophoresis. The PAGE gel was run at 250 V for 25 min in 1×TBE. The RNA was stained with SYBR Gold (Thermo Fisher Scientific, Waltham, MA, USA) and detected using an LAS-3000 instrument.

3-2-11. Cytokine release from DC2.4 cells by CpG-exo.

The evaluation of cytokine release from DC2.4 cells added to CpG-exo was performed as previously described [63]. Briefly, CpG-exo stored for 1 week at -80 °C or at room temperature after lyophilization with 50 mM trehalose were diluted to the indicated concentration using Opti-MEM (Thermo Fisher Scientific) and were added to DC2.4 cells. DC2.4 cells treated with

lipopolysaccharide (LPS) or Opti-MEM alone were prepared as a positive and negative control, respectively. The cells were incubated at 37 °C for 4 hours, after which the supernatants were collected. The levels of tumor necrosis factor (TNF)-α and interleukin (IL)-6 in the supernatant were determined by enzyme-linked immunosorbent assay (ELISA) using OptEIA™ sets (Pharmingen, San Diego, CA, USA).

3-2-12. Statistics

Differences were evaluated using Student's t-test and were considered statistically significant at $p < 0.05$.

3-3. Results

3-3-1. The addition of trehalose prevented the aggregation of exosomes during lyophilization

To investigate the effect of lyophilization on the morphology of exosomes, exosomes stored at -80 °C or at room temperature for 1 week after lyophilization with or without trehalose were observed using TEM (Fig. 15). TEM images showed that exosomes lyophilized without the cryoprotective agent trehalose formed aggregations, indicating that lyophilization without cryoprotectant damaged the exosomes. On the other hand, TEM images showed that lyophilization with trehalose did not cause the aggregation of exosomes and did not change their morphology compared to those stored at -80 °C. The PDI of exosomes lyophilized without cryoprotectant was wider than the others indicating that there were large aggregations in the sample (Fig. 15d). The PDI of exosomes lyophilized with trehalose were comparable to those stored at -80 °C. Zeta potentials of the exosomes were similar under all the storage conditions (Fig. 15e). These results indicate that trehalose could prevent the aggregation of exosomes during lyophilization. Since these results showed that lyophilization without trehalose damaged the

exosomes, I did not perform further studies of exosome samples lyophilized without cryoprotectant.

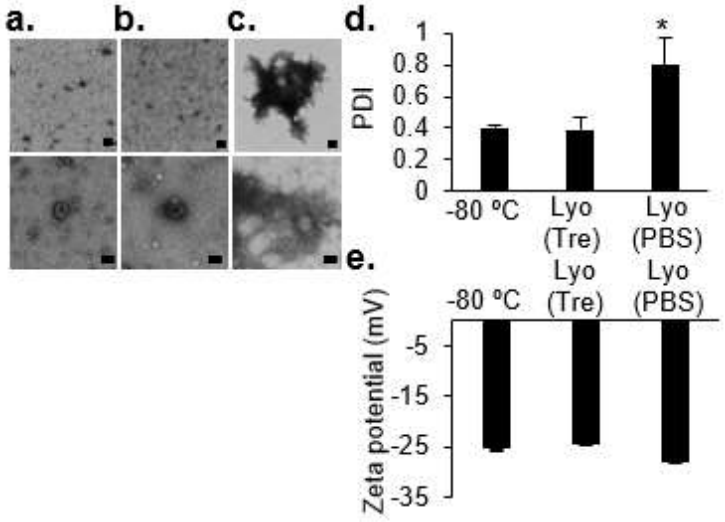

Figure 15. Trehalose has a lyoprotective effect on the physical properties of exosomes. (a-c) TEM images of exosomes stored at (a) -80 ºC and room temperature after (b) lyophilization with or (c) without trehalose. Scale bar = 500 nm for upper panel and 100 nm for lower panel (d) The poly dispersity index (PDI) of exosomes at the indicated storage conditions. (e) Zeta potential of exosomes stored at the indicated conditions. The data are expressed as the mean ± standard deviation (n = 3).

3-3-2. Protein and RNA contents were preserved in the lyophilized exosomes even after storage at room temperature

Western blot analysis showed that exosomal markers, i.e. alix, HSP70, and CD81, were contained in both the exosomes stored at -80 °C and the lyophilized exosomes stored at room temperature for 1 week (Fig. 16a). PAGE analysis of exosomal protein and RNA samples showed that those in the lyophilized exosomes stored at room temperature

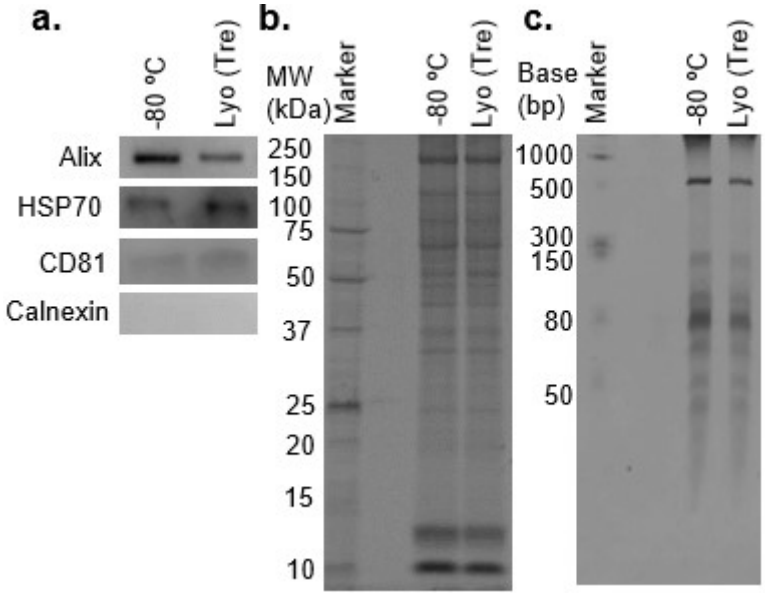

Figure 16. Effect of lyophilization on exosomal protein and RNA content. (a) Western blots of Alix, HSP70, CD81 and calnexin in exosomes stored at -80 °C (lane 1) and at room temperature after lyophilization with trehalose (lane 2). (b) Lumitein staining of exosomal proteins after SDS-PAGE. Lane 1: marker. Lane 2: exosomes stored at -80 °C. Lane 3: exosomes stored at room temperature after lyophilization with trehalose. (c) PAGE analysis of exosomal RNA. Lane 1: marker. Lane 2: exosomes stored at -80 °C. Lane 3: exosomes stored at room temperature after lyophilization with trehalose.

were comparable to those stored at -80 °C (Fig. 16b, c), indicating that exosomal protein and RNA were not damaged by lyophilization in the presence of trehalose. These data suggest that lyophilized exosomes can be stored at room temperature for 1 week without any apparent changes in exosomal protein and RNA content.

3-3-3. Lyophilization can preserve activity of CpG exosomes

Next, I investigated whether lyophilization could be exploited to preserve the function of exosomes loaded with functional nucleic acids. As a functional nucleic acid, I used CpG-exo to stimulate DC2.4 cells. It was found that after stimulating DC2.4 with CpG exosomes stored at -80 °C or at room temperature after lyophilization for 1 week, the levels of TNF-α were 13.9 ± 0.8, 13.8 ± 1.7 ng/ml, respectively and levels of IL-6 were 814 ± 175, 828 ± 160 pg/ml, respectively. There was no significant difference between these two groups in the amount of TNF-

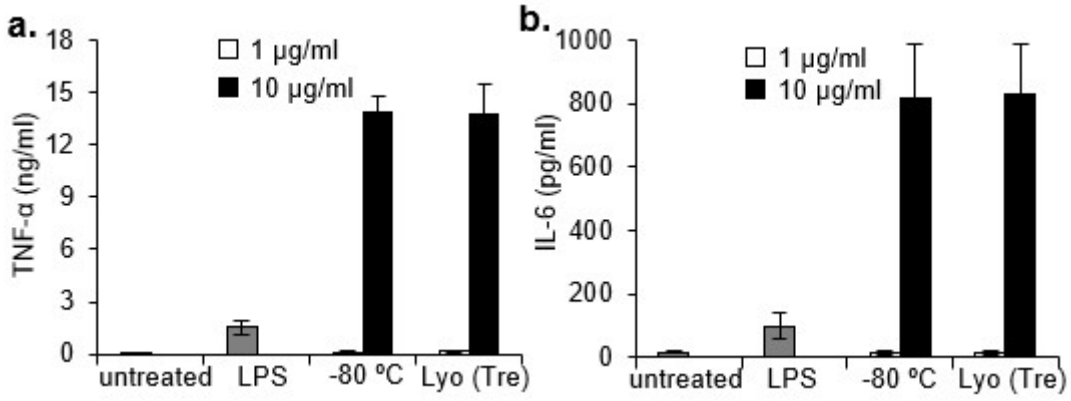

Figure 17. Stimulation of DC2.4 dendritic cells with CpG exosomes stored at -80 °C or at room temperature after lyophilization. The concentration of TNF-α (a) and IL-6 (b) in the culture supernatant of DC2.4 cells after stimulation with CpG-exo stored at the indicated conditions at final concentrations of 1 and 10 μg/ml. LPS (1 ng/ml) was used as a positive control. The results are expressed as the mean ± standard deviation (n = 4).

α and IL-6.

3-3-4. Luciferase activity was retained in lyophilized gLuc-LA exosomes after storage at room temperature

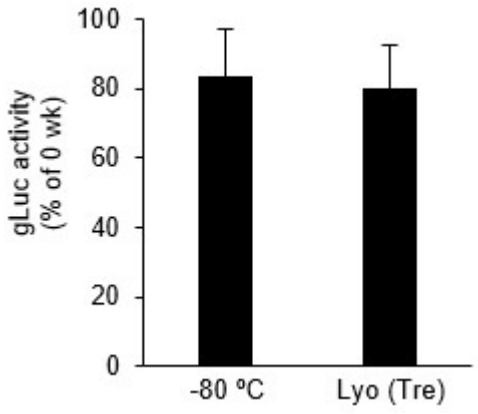

Figure 18. Exosomes labeled with gLuc-LA were stable after lyophilization. After lyophilization, luciferase activity of gLuc-LA exosomes was evaluated. The data are expressed as the mean ± standard deviation (n = 3).

To evaluate whether the function of the proteins loaded to exosomes was retained after lyophilization, gLuc-LA labeled exosomes were used. Luciferase activity of gLuc-LA exosomes stored at -80 °C or at room temperature after lyophilization for 4 weeks was measured. gLuc activity of exosomes stored at -80 °C and at room temperature after lyophilization was comparable, which was 83.3 ± 14.1 and 79.8 ± 12.6 % respectively. This result demonstrated that gLuc-LA labeled exosomes stored at room temperature after lyophilization retained luciferase activity at a comparable level to exosomes stored at -80 °C.

3-3-5. Lyophilization had little effect on the pharmacokinetics of exosomes

gLuc-LA exosomes stored at the indicated conditions for 4 weeks were intravenously injected into mice. I found that serum luciferase activity rapidly decreased regardless of the storage conditions (Fig. 19a), indicating that lyophilization had little effect on the pharmacokinetics of exosomes. Fig. 19b-d shows the following pharmacokinetic parameters of exosomes: the AUC, MRT and $t_{1/2\alpha}$. The AUC of the exosomes stored at -80 °C and at room temperature after lyophilization were 0.765 ± 0.393 and 0.791 ± 0.182 % of the dose•h/ml, respectively. The MRT of exosomes stored at -80 °C and at room temperature after lyophilization were 0.257 ± 0.022 and 0.304 ± 0.016 hours, respectively. The $t_{1/2\alpha}$ of exosomes stored at -80 °C and at room temperature after lyophilization were 2.27 ± 0.53 and 2.48 ± 0.40 min, respectively. These parameters were not remarkably different between storage conditions.

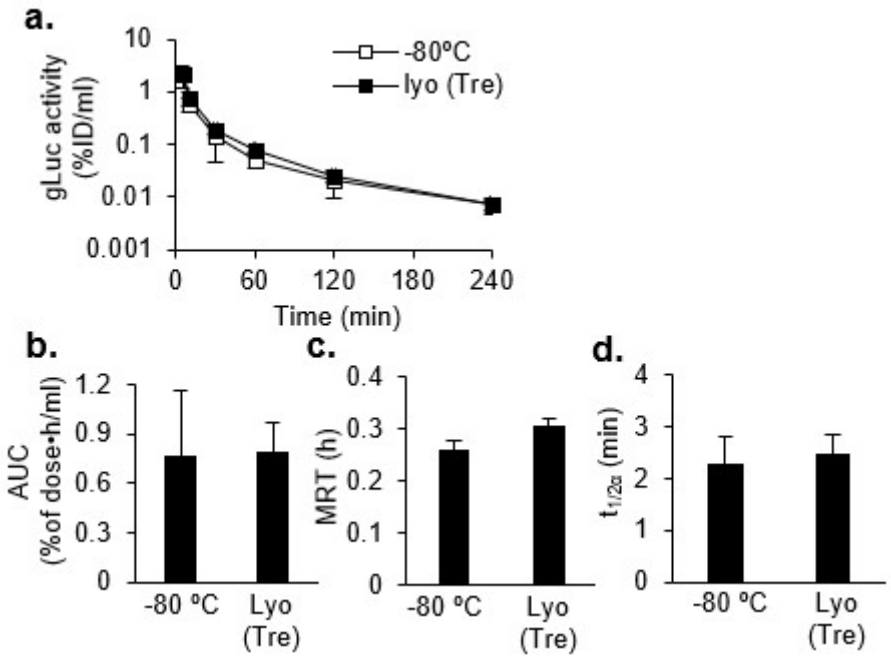

Figure 19. Time-course of serum gLuc activity after intravenous injection of exosomes stored at -80 °C and at room temperature after lyophilization. (a) Serum gLuc activity was measured after intravenously injecting exosomes stored at -80 °C (opened box) and at room temperature after lyophilized with trehalose (closed box). The results are expressed as the mean ± standard deviation (n = 4). % ID: % of injected dose. The pharmacokinetic parameters of exosomes stored at the indicated condition after intravenous injection are: (b) AUC, (c) MRT, (d) $t_{1/2\alpha}$, shown as histograms.

3-4. Discussion

In the present study, I evaluated the effect of lyophilization on the preservation of exosomes. TEM images (Fig. 15a, c.) showed that lyophilization of exosomes in the absence of trehalose caused aggregation due to various stresses generated during the lyophilization steps. For example, the lipid bilayer may be damaged by ice crystals during freezing, vesicle fusion may occur during dehydration, or phase transitions may occurs during rehydration [57–59]. A study by Akers et al. investigated the effect of storage conditions on cerebrospinal fluid-derived extracellular vesicles (EV) and found that lyophilization without cryoprotectants caused a reduction in EV particle number likely due to the aggregation found in this current study [64]. The results of that study and this current study suggests that lyophilization without cryoprotectants damage the exosomes.

In the present study, it was shown that the addition of trehalose, a cryoprotectant, during lyophilization protected exosomes from damages, such as particle aggregation during

lyophilization, which is in agreement with another report demonstrating that cryoprotectants can prevent the aggregation of lipid particles during lyophilization [61]. Regarding the cryoprotective activity of trehalose, it has been reported that sugars replace water molecules around the lipid headgroup through the interaction between the phospholipid head group and OH moiety of sugar, which occurs during lyophilization. In addition, the glass matrix of sugar could prevent vesicle aggregation and reduce the damage caused by ice crystals [58,59].

Trehalose is a nonreducing homodisaccharide in which two glucose units are linked together in an α-1,1-glycosidic linkage. Trehalose is synthesized in several organisms to protect the structural integrity of cells when they are exposed to environmental stress [65]. It is widely used in a number of applications, including food, cosmetic, medical, and pharmaceutical [65,66]. Trehalose was also used in the exosome research field. It has been reported that the addition of 25 mM trehalose to exosome-like vesicles narrows the particle size distribution and increases the number of individual particles per microgram of protein. Moreover, trehalose can prevent aggregation of vesicles, caused by repeated freeze-thaw cycles [67]. Furthermore, the addition of 50 mM of trehalose minimized the aggregation of exosomes and maintained the original exosome size following electroporation [68]. Therefore, trehalose might be used not only as a cryoprotectant in exosome lyophilization, but also to protect the particle characteristics of exosomes.

I evaluated exosomal proteins and RNA, as well as their pharmacokinetics. The results indicated that lyophilization of exosomes in the presence of trehalose showed similar exosomal markers and protein and RNA profiles compared to those stored at -80 °C. Moreover, lyophilization with trehalose did not alter the pharmacokinetics of exosomes. These results indicate that lyophilization could preserve the endogenous content of exosomes as well as the physicochemical and pharmacokinetic properties of exosomes. Therefore, lyophilization may be utilized to preserve exosomes for applications such as biomarkers and drug delivery vehicles.

The immunostimulatory activity of CpG-exo was preserved after lyophilization with trehalose, suggesting that lyophilization with trehalose could be used to preserve DNA-loaded exosomes without the loss of activity of loaded DNA. Although there is controversy regarding

immunological activity of trehalose, as some reports showed anti-inflammatory effects of trehalose [69,70] and others did not [71], these findings showed that the addition of trehalose had little effect on the immunostimulatory activity of CpG-exo.

As gLuc activity of gLuc-LA-labeled exosomes was retained after lyophilization with trehalose, lyophilization also preserves enzyme activity of proteins loaded to exosomes. As exosomes were expected to become delivery carriers for various proteins such as catalase [72], lyophilization may be utilized to preserve exosomes loaded with pharmacologically active functional proteins.

In Chapter 3, I have developed room temperature preservation method of exosome using lyophilization in the presence of trehalose. Lyophilization could preserve exosome without degrading exosomal proteins and RNAs and without altering physicochemical and pharmacokinetic properties. Furthermore, using lyophilization, biologically active molecules loaded into exosome could be preserved at room temperature for 1 week. Since these results were conducted after storage less than one month, further investigation is required to evaluate long shelf-life of exosomes after lyophilization. To my knowledge, this is the first study demonstrating that lyophilization could be used to preserve exosomes at room temperature, which is of importance for the therapeutic application of exosomes.

Summary

Exosomes have potential to be used as drug delivery carriers. To exploit exosomes as drug delivery carriers, I have evaluated the pharmacokinetic and pharmaceutical characteristics of exosomes for the development of exosome-based drug delivery carrier over these 3 chapters.

Chapter 1: Evaluation of cell type-specific and common characteristics of exosomes derived from mouse cell lines

I demonstrated that the five different cell types produced different yields of exosomes. All of the exosomes produced were comparable physicochemical and pharmacokinetic properties after intravenous injection into mice. These results imply that it is desirable to select various types of exosome-producing cells from the viewpoint of productivity, such as exosome yield and ease of handling, when developing exosome-based DDS.

Chapter 2: Evaluation of the role of exosome surface proteins in the pharmacokinetics of exosome

I demonstrated that Gag protein can be used for labeling the inner surface of exosome and that untreated or ProK-treated exosomes showed comparable physicochemical properties and slightly different pharmacokinetics. Moreover, I found that surface proteins play some roles in pharmacokinetics of exosomes at the whole-body level in mice.

Chapter 3: Development of preservation method of exosomes at room temperature by using lyophilization.

I demonstrated that trehalose could be used to prevent exosomal damage during lyophilization. Moreover, it was found that the storage of lyophilized exosomes at room temperature did not affect protein and RNA content, physicochemical and pharmacokinetics

properties, or the function of protein and DNA loaded on exosomes. These findings may provide useful information about preservation method of exosome for using exosome as DDS.

In conclusion of my thesis, I demonstrated that types of cells poorly affected the physicochemical and pharmacokinetics of exosomes. I also found that surface proteins of exosomes play a role in the pharmacokinetics of exosomes. Moreover, I developed room temperature preservation method of exosomes using lyophilization in the presence of cryoprotectant. The findings of this thesis may provide useful information for the development of exosome-based drug delivery systems.

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List of Publications

Cell type-specific and common characteristics of exosomes derived from mouse cell lines: Yield, physicochemical properties, and pharmacokinetics.

Chonlada Charoenviriyakul, Yuki Takahashi, Masaki Morishita, Akihiro Matsumoto, Makiya Nishikawa, Yoshinobu Takakura.

European Journal of Pharmaceutical Sciences 2017, 96, 316–322

Role of Extracellular Vesicle Surface Proteins in the Pharmacokinetics of Extracellular Vesicles

Chonlada Charoenviriyakul, Yuki Takahashi, Masaki Morishita, Makiya Nishikawa, Yoshinobu Takakura.

Molecular Pharmaceutics 2018, 15, 1073−1080

Preservation method of exosomes at room temperature by using lyophilization.

Chonlada Charoenviriyakul, Yuki Takahashi, Makiya Nishikawa, Yoshinobu Takakura.

Manuscript submitted

List of Other Publications

Macrophage-dependent clearance of systemically administered B16BL6-derived exosomes from the blood circulation in mice

Takafumi Imai, Yuki Takahashi, Makiya Nishikawa, Kana Kato, Masaki Morishita, Takuma Yamashita, Akihiro Matsumoto, Chonlada Charoenviriyakul, Yoshinobu Takakura

Journal of Extracellular Vesicles 2015, 4, 26238

Role of Phosphatidylserine-Derived Negative Surface Charges in the Recognition and Uptake of Intravenously Injected B16BL6-Derived Exosomes by Macrophages

Akihiro Matsumoto, Yuki Takahashi, Makiya Nishikawa, Kohei Sano, Masaki Morishita, Chonlada Charoenviriyakul, Hideo Saji, Yoshinobu Takakura

Journal of Pharmaceutical Sciences 2017, 106, 168-175

Accelerated growth of B16BL6 tumor in mice through efficient uptake of their own exosomes by B16BL6 cells

Akihiro Matsumoto, Yuki Takahashi, Makiya Nishikawa, Kohei Sano, Masaki Morishita, Chonlada Charoenviriyakul, Hideo Saji, Yoshinobu Takakura

Cancer Sciences 2017, 108, 1803–1810

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