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Augmented liver targeting of exosomes by surface modification with cationized pullulan

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ABSTRACT

Exosomes are membrane nanoparticles containing biological substances that are employed as therapeutics in experimental inflammatory models. Surface modification of exosomes for better tissue targetability and enhancement of their therapeutic ability was recently attempted mainly using gene transfection techniques. Here, we show for the first time that the surface modification of exosomes with cationized pullulan, which has the ability to target hepatocyte asialoglycoprotein receptors, can target injured liver and enhance the therapeutic effect of exosomes. Surface modification can be achieved by a simple mixing of original exosomes and cationized pullulan and through an electrostatic interaction of both substances. The exosomes modified with cationized pullulan were internalized into HepG2 cells *in vitro* to a significantly greater extent than unmodified ones and this internalization was induced through the asialoglycoprotein receptor that was specifically expressed on HepG2 cells and hepatocytes. When injected intravenously into mice with concanavalin A-induced liver injury, the modified exosomes accumulated in the liver tissue, resulting in an enhanced anti-inflammatory effect *in vivo*. It is concluded that the surface modification with cationized pullulan promoted accumulation of the exosomes in the liver and the subsequent biological function, resulting in a greater therapeutic effect on liver injury.

Statement of significance

Exosomes have shown potentials as therapeutics for various inflammatory disease models.
This study is the first to show the specific accumulation of exosomes in the liver and enhanced anti-inflammatory effect via the surface modification of exosomes using pullulan, which is specifically recognized by the asialoglycoprotein receptor (AGPR) on HepG2 cells and hepatocytes. The pullulan was expressed on the surface of PKH-labeled exosomes, and it led increased accumulation of PKH into HepG2 cells, whereas the accumulation was canceled by AGPR inhibitor. In the mouse liver injury model, the modification of PKH-labeled exosomes with pullulan enabled increased accumulation of PKH specifically in the injured liver. Furthermore the greater therapeutic effects against the liver injury compared with unmodified original exosomes was observed.

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1. Introduction

Exosomes are defined as membrane nanoparticles released via the fusion of multivesicular bodies with the plasma membrane [1]. They contain various biologically active substances and play an important role in intercellular communication [2,3]. A representative example of these factors is paracrine factors secreted from mesenchymal stem cells (MSCs). Studies on several animal models have reported that the exosomal fraction present in conditioned medium of MSCs culture has therapeutic effects [2,4–6]. This unique property has encouraged the application of exosomes as therapeutic molecules [7–9]. Furthermore, an approach to modify the surface of exosomes for better tissue targetability and efficient delivery of their contents into the cytosol has recently been reported, which would be particularly promising when used in combination with genetic engineering [10–12].

The objective of this study is specific delivery of MSC-derived exosomes into the liver after their injection. It has been reported that they are internalized by macrophages after their injection and then express their effects through the immunological function

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of macrophages [13–15]. If MSC-derived exosomes readily accumulate in injured liver and are internalized by cells other than macrophages, they could exert their effects more directly on the tissue, conferring greater potential for therapeutic and diagnostic purposes [14].

Pullulan is a maltotriose consisting of three glucose moieties that are connected by an α-1,4 glycosidic bond [16,17]. It is specifically internalized by HepG2 cells and hepatocytes through an asialoglycoprotein receptor (ASGPR) and is an efficient carrier of siRNA for specific delivery to hepatocytes [18,19]. In a previous study, modification with pullulan enabled the efficient accumulation of drugs in parenchymal hepatocytes and consequently enhanced their therapeutic effect in the liver [17].

Building on this previous work, in this study, exosomes were modified with pullulan by simple mixing to enhance their liver accumulation and therapeutic effect on a liver injury. Pullulan was cationized with spermine, a polyamine present in the body, to enhance its electrostatic interaction with the negatively charged surface of original exosomes [20]. The enhanced internalization of exosomes modified with cationized pullulan and its pathway was evaluated in an *in vitro* study, and the enhanced accumulation and therapeutic effect were confirmed *in vivo* in a concanavalin A-induced liver injury model, established as described previously [21]. The therapeutic effect of the modified exosomes and the underlying mechanisms were evaluated by histological and immunological analyses.

2. Materials and methods

2.1. Animals

All animal experiments were carried out in accordance with procedures approved by the Animal Experimentation Committee of the Institute for Frontier Medical Sciences (approval number: #F173). Male C57B6 mice were obtained from Shimizu Laboratory Supplies Co., Ltd. (Kyoto, Japan). All mice were maintained under specific pathogen-free conditions in the animal facility at the Institute for Frontier Medical Sciences.

2.2. Collection of exosomes

Exosomes were purified from the supernatant of MSCs culture. Collection was carried out by a slightly modified version of a previously reported procedure (Fig. 1a) [22,23]. The supernatant was centrifuged twice at 2000*g* and 10,000*g* to exclude cellular or other debris. The final supernatant was then passed through 0.45-μm pore filters (Merck Millipore, Billerica, MA, USA) and the filtrate was used for the collection of exosomes. The exosome fraction in the filtrate was pelleted by ultracentrifugation at 100,000*g* for 60 min with a Hitachi CP80WX ultracentrifuge (Hitachi Koki Co., Ltd., Tokyo, Japan). The pellet was resuspended in PBS and similarly ultracentrifugated again. Collected exosomes were dispersed in phosphate-buffered saline (PBS) and the amount of exosomes collected was determined by measuring the protein concentration using the BCA Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA), in accordance with the manufacturer's protocol [22]. Ultracentrifuge tubes and collection bottles for exosomes were sterilized prior to use, and the collection was carried out in a sterilized manner. Collected exosomes were stored in the refrigerator at 4 °C for up to a week before used for experiments.

2.3. Analyses of the size and zeta potential of collected exosomes

The diameter of collected exosomes dispersed in PBS was determined using the qNano system (Izon, Christchurch, New Zealand). Changes in the diameter and zeta potential of exosomes modified

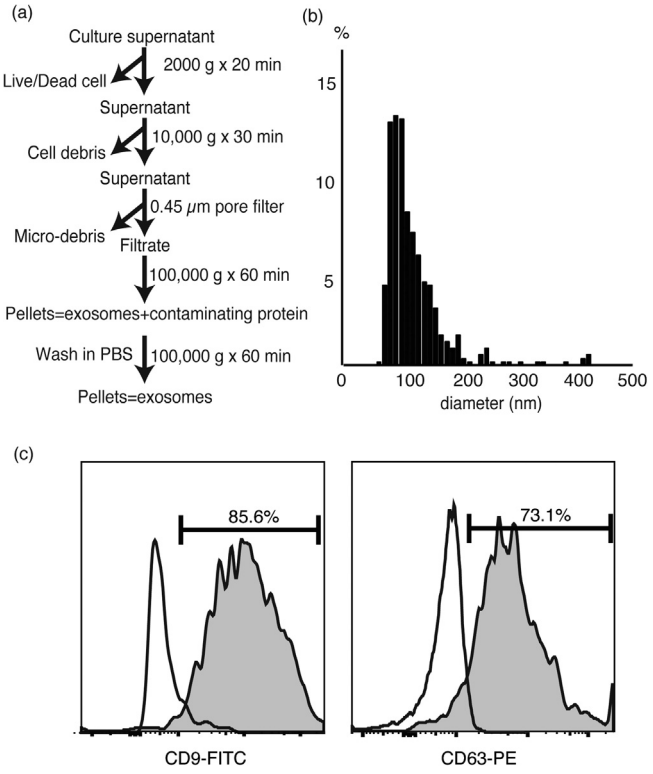

Fig. 1. Collection and characteristics of exosomes. (a) An overview of the collection procedure. Collected exosomes were evaluated for their diameter (b), and the fraction of typical surface markers of exosomes, CD9 and CD63, by flowcytometric analysis (c).

with pullulan into which spermine had been introduced at 6.0, 11.6, and 17.0 mol% were measured with a Zetasizer Nano ZS with He-Ne laser 633 nm wavelength (Malvern Instruments, Malvern, UK) at 20 °C. This measurement was repeated 100 times for each sample and the result is expressed as the mean. The experiment was repeated three times.

2.4. Preparation of cationized pullulan derivatives and their reactions with collected exosomes

Pullulan with a weight-average molecular weight of 47,300 (Hayashibara, Okayama, Japan) and spermine (Sigma Chemical, St. Louis, MO, USA) were used for a preparation of cationized pullulan. Spermine was introduced to the hydroxyl groups of pullulan by an N,N′-carbonyldiimidazole (CDI) activation method [18,24]. Spermine and CDI were added to 50 ml of dehydrated dimethyl sulfoxide containing 50 mg of pullulan. Following agitation using a magnetic stirrer at 35 °C for 20 h, the reaction mixture was dialyzed against ultrapure double-distilled water for 2 days using a dialysis membrane with a molecular weight cut-off of 12,000–14,000 (Viskase Companies Inc., Willowbrook, IL, USA). Then, the dialyzed solution was freeze-dried to obtain samples of cationized pullulan. The rate of spermine introduction was determined by conventional elemental analysis and expressed by the molar percentage of spermine introduced onto the hydroxyl groups of pullulan. Collected exosomes were mixed with the cationized pullulan, followed by leaving the mixed solution for 15 min at room temperature.

2.5. Detection of cationized pullulan on the surface of modified exosomes

The presence of cationized pullulan on the surface of exosomes was evaluated by the lectin-induced aggregation method [25,26]. A

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