Biochemical and Biophysical Research Communications xxx (2017) 1–7

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Glycan profiling analysis using evanescent-field fluorescence-assisted lectin array: Importance of sugar recognition for cellular uptake of exosomes from mesenchymal stem cells

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ARTICLE INFO

Article history:
Received 13 July 2017
Accepted 22 July 2017
Available online xxx

Keywords:
Exosome
Mesenchymal stem cell
Glycan
Lectin array
Sialic acid

ABSTRACT

Studies involving the functional analysis of exosomal contents including proteins, DNA, and RNA have been reported. Most membrane proteins and lipids are glycosylated, which controls their physical properties and functions, but little is known about glycans on exosomes owing to the difficulty of analysing them. To shed light on these issues, we collected exosomes from mesenchymal stem cells (MSCs) derived from human adipose tissue for glycan profiling using evanescent-field fluorescence-assisted lectin array as well as analysis of their uptake *in vivo*. Initial analyses showed that the mean diameter of the collected exosomes was 178 nm and they presented with typical exosomal and MSC markers. Regarding the glycan profiling, exosomes interacted more strongly than the membrane of the original MSCs did with a range of lectins, especially sialic acid-binding lectins. The findings also showed that cellular exosome uptake involved recognition by HeLa cell-surface-bound sialic acid-binding immunoglobulin (Ig)-like lectins (siglecs). Confirming this siglec-related uptake, *in vivo* experiments involving subcutaneous injection of the fluorescently labelled exosomes into mice showed their transport into lymph nodes and internalization by antigen-presenting cells, particularly those expressing CD11b. Closer analysis revealed the colocalization of the exosomes with siglecs, indicating their involvement in the uptake. These findings provide us with an improved understanding of the importance of exosomal transport and targeting in relation to glycans on exosomal surfaces, potentially enabling us to standardize exosomes when using them for therapeutic purposes.

1. Introduction

Extracellular vesicles such as exosomes, which are nanosized vesicles originating from endosomes, play an important role in cell–cell signalling [1,2]. Exosomes deliver functional molecules including nucleic acids [DNA, mRNA, and microRNA (miRNA)] and proteins into recipient cells, and produce both beneficial and detrimental effects [3,4]. For example, tumour-derived exosomes promote tumour progression, angiogenesis, and metastasis by their growth factors and miRNA [5], and mesenchymal stem cell (MSC)-derived exosomes have attracted growing interest as a new type of therapeutic agent [6–8]. As the characteristics of cells can be conveyed elsewhere via the cargo of exosomes, having various influences on the surroundings, exosomes have attracted attention as biological drug delivery carriers in recent years [9–11]. However, the mechanisms by which their contents are transferred and by which their selectivity for target cells is achieved are still not understood.

Glycans in glycolipids and glycoproteins play numerous essential roles in cells, including in protein folding, stability, cell adhesion, regulation of cell differentiation, and proliferation [12]. Thousands of glycan combinations are possible according to the types of sugars and their bonding form, leading to different physical properties and functions, even for the same types of glycolipids or glycoproteins [13]. Furthermore, protein–glycan interactions on

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http://dx.doi.org/10.1016/j.bbrc.2017.07.126
0006-291X/

the cell are involved in the control of immune reactions and pathogen adhesion and entry, indicating that glycans also function as cell-targeting ligands [14,15]. Based on this background, exosomal glycans should play essential roles in various processes related to extracellular vesicles, such as their origin, uptake into recipient cells, and protein sorting. However, few studies have examined the interactions between glycans on the surface of exosomes and cells [16,17], although the identity and functions of nucleic acids and proteins in exosomes have been extensively studied. This is mainly because the analytical methods required to determine glycan structures are more complicated than those for other components owing to their structural complexity. The typical methods applied in glycan analysis are chromatography, mass spectrometry (MS), and lectin blotting [18,19]. Specifically, MS-based analysis is commonly used because of its several advantages, including a broad range of applications for complexed glycan, site-specific glycan analysis, and high sensitivity [20]. However, these methods cannot directly obtain information on the glycans on the surfaces of intact exosomes without their disassembly. In MS measurement, for example, pretreatments of glycolipids or glycoproteins, such as hydrolysis, enzymatic digestion, and reduction, are required before analysis [21,22]. Lectin array analysis is superior for determining the profiles of glycan on the surface of exosomes. However, few studies involving glycan profiling analysis of exosomes from cancer cells [23–25] or urine [26,27] have been reported. In the conventional lectin array system, a fluorescence scanner is used to detect the fluorescently labelled glycoproteins; however, washing steps to remove unbound samples lead to low sensitivity owing to weak interactions between lectin and glycan ($K_d = 10^{-4}$–10^{-7} M) [28]. To avoid this inconvenience, Kuno et al. developed an innovative system based on evanescent-field fluorescent detection in which the evanescent wave could excite a fluorescent field in the vicinity of a glass slide (less than 200 nm), indicating that it enables highly sensitive and real-time detection without washing steps [29]. We apply here, for the first time, an evanescent-field fluorescence-assisted (EFF) lectin array system for analysing glycan profiles of intact exosomes. A remarkable feature of this method is its ability to directly detect relatively weak glycan–lectin interactions in the liquid phase without the destruction of exosomes.

In this study, glycan profiling of exosomes from human adipose-derived mesenchymal stem cells (ADSCs) was performed using an EFF lectin array system (glass slides spotted with 45 lectins). Recently, MSC-derived exosomes have attracted growing attention as an alternative option for regenerative therapy because of several advantages, including stability, longer storage, and less possibility of immune rejection [8,9,30]. We found that the difference of glycan profile between the exosome and the membrane of the original MSCs was detected with high sensitivity. In particular, the exosomes were shown to interact with sialic acid-binding lectins and the sialic acids on exosomes were found to interact with sialic acid-recognizing lectins on the cell surface *in vitro* and *in vivo*. This is the first report of the glycan profiling of exosomes from MSCs and also of the specific interaction of exosomes with cells via sialic acid-recognizing lectins. Exosomal glycan analysis provides useful information about the characteristics of their cell of origin and exosome–cell interactions.

2. Materials and methods

2.1. Cell cultures

ADSCs were purchased from Lonza (Walkersville, MD, USA) and cultured in Adipose-Derived Stem Cell Growth Medium BulletKit™ (Lonza) at 37 °C in an atmosphere containing 5% CO_2. HeLa cells were purchased from JCRB Bank (Japanese Collection of Research Biosources 9004, Osaka, Japan) and cultured in Minimum Essential Medium (Thermo Fisher Scientific, Waltham, MA, USA) containing 1% nonessential amino acids, 100 IU/mL penicillin and 100 μg/mL streptomycin, and 10% foetal bovine serum at 37 °C in an atmosphere containing 5% CO_2.

2.2. Isolation of exosomes from ADSC culture supernatants

Upon reaching 70%–80% confluence, the cell culture medium was replaced with StemPro® MSC SFM (Thermo Fisher Scientific) containing CTS™ GlutaMAX™-I and then exosomes were isolated by differential centrifugation after 48 h of incubation. The medium was centrifuged at 300 × *g* for 10 min, 2000 × *g* for 10 min, and 10,000 × *g* for 30 min to remove dead cells and cell debris. The resulting supernatants were centrifuged at 120,000 × *g* for 100 min at 4 °C using a 50.2 Ti rotor (Beckman Coulter). Exosome pellets were washed with phosphate-buffered saline (PBS) and resuspended in PBS for further analysis. Protein concentrations of exosomes were estimated using a Micro BCA assay kit (Pierce, Rockford, IL, USA).

2.3. Nanoparticle tracking analysis (NTA)

The size distribution of exosomes was determined by Nanoparticle Tracking Analysis (NTA) using a NanoSight LM10 (NanoSight, Amesbury, UK) with a blue laser. The concentration of exosomes was diluted to about 10^8–10^9 particles/mL for analysis.

2.4. Western blotting

Protein expression levels of MSC and exosome markers (CD73 and CD81) were determined by western blotting. Cell lysates and exosomes were separated by 12.5% SDS-PAGE and transferred onto polyvinylidene difluoride membranes. After blocking with Blocking-One (Nacalai Tesque Inc., Kyoto, Japan) for 30 min, membranes were blotted with the following primary antibodies: anti-CD73 (EPR6114; GeneTex Inc., Irvine, CA, USA) and anti-CD81 (Thermo Fisher Scientific). The membranes were then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies and ECL Western Blotting Detection Reagents (GE Healthcare). The bands were visualized using LAS-4000 (GE Healthcare).

2.5. Transmission electron microscopy (TEM)

The morphologies of exosomes were observed using an HT7700-TEM (Hitachi, Tokyo, Japan) at an accelerating voltage of 100 kV, as previously described [31].

2.6. Lectin array

ProteoExtract® Subcellular Proteome Extraction Kit (Calbiochem, Merck, Darmstadt, Germany) was used to prepare membrane fractions from ADSCs. ADSCs and ADSC exosomes were labelled with Cy3 Mono-Reactive dye pack (GE Healthcare, Little Chalfont, UK), and both samples (31–2000 ng/mL) were applied to each well of a lectin microarray chip (LecChip™; GlycoTechnica, Yokohama, Japan). After overnight incubation at 37 °C, fluorescence intensity was measured using GlycoStation™ Reader 1200 (GlycoTechnica) and the data were analysed using GlycoStation®Tools Pro Suite 1.5 (GlycoTechnica). The experiments were performed in duplicate (ADSCs) or triplicate (ADSC exosomes).

Please cite this article in press as: A. Shimoda, et al., Glycan profiling analysis using evanescent-field fluorescence-assisted lectin array: Importance of sugar recognition for cellular uptake of exosomes from mesenchymal stem cells, Biochemical and Biophysical Research Communications (2017), http://dx.doi.org/10.1016/j.bbrc.2017.07.126