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Physical characterization and profiling of airway epithelial derived exosomes using light scattering

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ABSTRACT

Exosomes and other extracellular vesicles have been gaining interest during the last decade due to their emerging role in biology and, disease pathogenesis and their biomarker potential. Almost all published research related to exosomes and other extracellular vesicles include some form of physical characterization. Therefore, these vesicles should be precisely profiled and characterized physically before studying their biological role as intercellular messengers, biomarkers or therapeutic tools. Using a combination of light scattering techniques, including dynamic light scattering (DLS) and multi-angle laser light scattering combined with size exclusion separation (SEC-MALLS), we physically characterized and compared distinct extracellular vesicles derived from the apical secretions of two different cultured airway epithelial cells. The results indicated that epithelial cells release vesicles with distinct physical properties and sizes. Human primary tracheobronchial cell culture (HTBE) derived vesicles have a hydrodynamic radius (*R*h) of approximately 340 nm while their radius of gyration (*R*g) is approximately 200 nm. Electron microscopy analysis, however, revealed that their spherical component is 40–100 nm in size, and they carry filamentous, entangled membrane mucins on their surface that increases their overall radius. The mucin decoration on the surface defines their size and charge as measured using light scattering techniques. Their surface properties mirror the properties of the cells from which they are derived. This may provide a unique tool for researchers to elucidate the unanswered questions in normal airway biology and innate and adaptive defense, including the remodeling of airways during inflammation, tumorigenesis and metastasis.

1. Introduction

Exosomes and other extracellular vesicles have been gaining interest during the last decade due to their emerging role in inter-cellular communication [1,2], immune modulation [3,4] and as potential diagnostic in various disease-related conditions [5–7]. Exosomes, enclosed by a phospholipid bilayer, originate in the late endosomal compartment from the inward budding of multi-vesicular bodies (MVBs) [8]. Using electron microscopy (EM), these MVBs were observed to bud from the plasma membrane, leading to the secretion of the internal vesicles into the extracellular environment [9,10]. Other extracellular vesicles, however, either fuse from plasma membrane or are shed from polarized epithelia e.g. microvilli [11,12]. Exosomes and other extracellular vesicles are generally isolated using differential centrifugation, column chromatography, filtration or polymeric precipitation and are characterized by a variety of methods including light scattering, particle tracking analysis and EM. Additionally, their cargo is identified via proteomics and miRNA/mRNA analysis. The physical and molecular characteristics of exosomes and other extracellular vesicles depends on the system from which they are derived. They are released into the extracellular space by various cell types including epithelial cells, immune cells, reticulocytes and tumor cells, and are also found in body fluids including saliva [13,14], urine [15], broncho-alveolar lavage fluid (BALF) [16], airway [17] and cervical mucus [18] plasma [19], and amniotic fluid [20]. They carry cargo that contains a distinct set of proteins [21], lipids [22], and RNA (miRNAs, mRNAs) [23]. These molecules, which may be a signature of their parent cell, can be horizontally transferred to neighboring cells and thus serve as mediators of intercellular signaling and cellular response.

Secretions from tracheobronchial epithelial cells contain hundreds of innate immune molecules that are released from different types of cells, e.g., secretory and ciliated. These cells also release

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exosomes and other extracellular vesicles that may be involved in diverse physiological processes in airway biology and in innate [17], and adaptive [24] immune response. Previous studies reported that there is increased vesicle release by bronchial epithelial cells in cystic fibrosis patients [25], and from the lungs of asthmatic patients which induced proliferation and chemotaxis of undifferentiated monocytes [26]. In our previous study, we demonstrated that exosomes derived from human HTBE epithelial cells could neutralize human influenza A virus [17] through their sialic acid moieties.

Research on exosomes and other secreted membrane vesicles has expanded in the last decade due to their putative role in therapeutics [27], disease pathogenesis [28] and biomarker discovery [29,30]. These vesicles should be precisely characterized biophysically prior to investigating their role as intercellular messengers, biomarkers or therapeutic tools. In this study, we physically characterized and compared the vesicles isolated from the apical secretions of two different cultured human primary airway epithelial cells (HTBE) and the Calu-3 cell line using three different techniques; dynamic light scattering (DLS), multi-angle laser light scattering combined with size exclusion chromatography (SEC-MALLS) and nanoparticle tracking analysis (NTA).

2. Material and method

2.1. Cell culture

Two different airway cell culture systems that secrete mucus were used in this study: human tracheobronchial epithelial cells (HTBE) and the airway epithelial Calu-3 cell line. Primary human airway epithelial cells were isolated and cultured as previously described in details [17,31]. Briefly, the cells were plated at a density of 600,000 cells/well on permeable Transwell-Col (T-Col; 24 mm diameter) support. HTBE cultures were generated by provision of an air–liquid interface for 4–6 week to form well-differentiated, polarized cultures that resemble in vivo pseudostratified mucociliary epithelium. Airway epithelial Calu-3 cells were derived from pleural effusion associated with a human lung adenocarcinoma. Calu-3 cells were grown on 24-mm Transwell supports (Corning Life Sciences, MA, USA) and maintained at the air–liquid interface for at least three weeks, as previously described [32,33]. Mucus secretions were obtained by performing two sequential 1 mL PBS washes on the apical surface of the cultures. Each wash was collected following a 30 min incubation at 37 °C. Culture washings obtained from 6 individual cultures were pooled and centrifuged at 3000*g* for 10 min to remove the dead cells. Washings were subsequently subjected to differential sedimentation to isolate the exosomes as described below.

2.2. Isolation of exosomes

Exosomes were isolated using differential centrifugation [17] from HTBE and Calu-3 secretions, which contain complex protein content [34] and are viscous in nature. Briefly, the pooled HTBE and Calu-3 secretions were diluted 1:1 with PBS and were centrifuged at 3000*g* for 10 min and 10,000*g* for 30 min to eliminate cell debris and other particles. The exosomal vesicles were subsequently pelleted at 65,000 g. The pellet was then washed with PBS and pelleted again at 100,000 g. This washing procedure was repeated to remove any protein or mucin contaminants, which are abundant in the HTBE/Calu-3 secretions. The isolated vesicles were resuspended in PBS and filtered through 0.22-μm filters to eliminate impurities and large-sized micro-particles and spun again at 100,000 g. Finally, the exosome pellets were resuspended in 50 μL of PBS and stored as 10 μL aliquots at −30 °C until further characterization analyses.

2.3. Characterization of exosomes

2.3.1. Dynamic light scattering

The size and zeta potential measurements were conducted using a Zetasizer Nano ZS system (Malvern Instruments, Malvern, U.K.). The dynamic light scattering technique analyzes the velocity distribution of particle movement by measuring the dynamic fluctuations of scattered light intensity at a fixed angle (173°) caused by the Brownian motion of the particle. It assesses the particle perpendicular to the light source at that instant, yielding the particle's hydrodynamic radius (*R*h), or diameter, calculated via the Stokes–Einstein equation [35]. DLS also uses a laser that passes through the sample to measure the velocity of the particles in an applied electric field of a known value called the electrophoretic mobility. For DLS measurements, 10 μL exosome aliquots were diluted in 990 μL of PBS (1:100) and then gently mixed to provide a homogeneous solution, and then 1 mL was transferred to a disposable cuvette for size measurements. For Zeta potential measurements, 10 μL exosome aliquot was diluted in 990 μL of water (1:100) and then transferred to a Malvern Clear Zeta Potential cell. Three independent aliquots were analyzed and three measurements were taken for each aliquots.

The data were acquired and analyzed using Dispersion Technology Software (DTS) (V7.01) supplied by the Malvern Zetasizer Nano-ZS. For the particle sizing in solution (DLS), the software provides multiple aspects and interpretations of the data collected for the sample such as intensity, volume, and number distribution graphs as well as a statistical analysis for each. The mean particle diameter is calculated from the particle distributions measured, and the polydispersity index (PdI) given is a measure of the size ranges present in the solution.

2.3.2. SEC-MALLS analysis

Ten microliter aliquots from the exosome preparations were diluted in 1000 μL PBS. A 500 μL aliquot was injected and chromatographed on a Sepharose CL-2B column (15 × 2.5 cm, GE healthcare life sciences) and eluted with 0.2 M NaCl at a flow rate of 500 μl/min. The column effluent was passed through an inline Dawn DSP laser photometer coupled to a Wyatt/Optilab 903 inferometric refractometer (Wyatt Technologies, Santa Barbara, CA, USA) to measure the molecular weight/radius of gyration and absolute sample concentration. Light scattering measurements were taken continuously at 18 angles between 15° and 151°; the captured data were integrated and analyzed using the Astra software provided with the Dawn photometer.

2.3.3. Nanoparticle tracking analysis (NTA)

The detailed characterization of exosomes using NTA is covered as another topic this issue of the methods. In this study we also used NTA for size and concentration analysis of the isolated exosomes using a NanoSight NS300 instrument equipped with NTA 3.0 analytical software. Each experiment was carried out in triplicate. Each sample was diluted in PBS (1:1000), and mixed before introduction into the sample chamber using a syringe pump with a constant flow rate. Three video recordings, of 60 s each were initiated. A combination of shutter speed and gain followed by manual focusing enables optimum visualization of a maximum number of vesicles. To accurately track the vesicles they were visualized as single points of light. The samples were advanced between each recording to perform replicate measurements. The NTA post-acquisition settings were optimized and kept constant between the samples, and each video was then analyzed to give