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Cellular uptake of extracellular vesicles is mediated by clathrin-independent endocytosis and macropinocytosis

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ABSTRACT

Recent evidence has established that extracellular vesicles (EVs), including exosomes and microvesicles, form an endogenous transport system through which biomolecules, including proteins and RNA, are exchanged between cells. This endows EVs with immense potential for drug delivery and regenerative medicine applications. Understanding the biology underlying EV-based intercellular transfer of cargo is of great importance for the development of EV-based therapeutics. Here, we sought to characterize the cellular mechanisms involved in EV uptake.

Internalization of fluorescently-labeled EVs was evaluated in HeLa cells, in 2D (monolayer) cell culture as well as 3D spheroids. Uptake was assessed using flow cytometry and confocal microscopy, using chemical as well as RNA interference-based inhibition of key proteins involved in individual endocytic pathways. Experiments with chemical inhibitors revealed that EV uptake depends on cholesterol and tyrosine kinase activity, which are implicated in clathrin-independent endocytosis, and on Na^+/H^+ exchange and phosphoinositide 3-kinase activity, which are important for macropinocytosis. Furthermore, EV internalization was inhibited by siRNA-mediated knockdown of caveolin-1, flotillin-1, RhoA, Rac1 and PAK1, but not clathrin heavy chain. Together, these results suggest that EVs enter cells predominantly via clathrin-independent endocytosis and macropinocytosis. Identification of EV components that promote their uptake via pathways that lead to functional cargo transfer might allow development of more efficient therapeutics through EV-inspired engineering.

1. Introduction

Extracellular vesicles (EVs) are cell-derived membranous nanoparticles that enclose and transport complex molecules, including proteins, nucleic acids, lipids and sugars, derived from the parent cell [1]. EVs have been found to function as natural carrier systems that efficiently deliver their molecular cargo to recipient cells. EVs are released by organisms ranging from prokaryotic cells to higher eukaryotes, and in mammals these vesicles have been reported to be released from almost all cell types. EVs are generally classified into three subtypes, apoptotic bodies, microvesicles and exosomes, according to their intracellular origin [2]. However, no uniform EV nomenclature exists as of yet, due to overlap in vesicle sizes and an absence of subtype-specific markers [3]. Therefore, vesicle subtypes are collectively referred to as EVs.

EVs have recently been demonstrated to act as a cell-to-cell communication system involved in the natural transfer of macromolecules between producer and recipient cells. As such, EVs can affect recipient cell behavior, and modify physiological as well as pathological processes [4]. For this reason, the potential usage of EVs for therapeutic applications, including cell-free approaches for tissue engineering and drug delivery, is increasingly being explored [5–7]. Very encouraging proof-of-concept studies suggest that EVs may bear intrinsic properties for anti-tumor immunotherapy [8], for treatment of immunological disorders (e.g. graft-versus-host disease [9]) or for protecting or regenerating tissue after e.g. kidney injury [10] or myocardial infarction [11]. Furthermore, EVs may be modified for targeted drug delivery, which has already been successfully demonstrated in small animal studies for therapeutic delivery of RNA [12], protein [13], and small molecules [14].

As EVs seem naturally capable of crossing biological barriers leading to functional delivery of their cargo, it may be hypothesized that they utilize native mechanisms for internalization and intracellular trafficking [15]. Endocytosis has been reported to be the major route through which EVs are engulfed by cells [16–19]. However, detailed mechanisms remain unknown. For successful utilization of EVs as therapeutic carrier systems, understanding the cellular entry routes that these endogenous carriers follow to deliver their cargo into recipient

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cells is of critical importance.

There are numerous mechanisms through which cells may internalize endocytic cargo. Generally, endocytosis is divided into two main subgroups: phagocytosis and pinocytosis. Phagocytosis is a type of endocytosis that involves internalization of relatively large (> 1 μm) particles and is typically restricted to specialized professional phagocytes. Pinocytosis, in contrast, is exhibited by all cells, and is commonly classified into clathrin-dependent endocytosis (CDE), clathrin-independent endocytosis (CIE) and macropinocytosis (MP). CIE can be further categorized into caveolae-, Arf-6, flotillin-1-, CDC42- and RhoA-dependent endocytosis [20,21].

The ability of nanoparticles to deliver their cargo and elicit a biological response is strongly determined by the mechanism through which they are taken up [22–24]. Here, we set out to identify the endocytic routes involved in internalization of EVs.

2. Materials and methods

2.1. Cell culture

A431 (human epidermoid carcinoma) cells (ATCC) and HeLa (human cervical carcinoma) cells (ATCC) were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 100 U/mL penicillin and 100 U/mL streptomycin. Cells were cultivated at 37 °C and 5% CO_2 under humidified conditions. Cells were routinely checked for *Mycoplasma* contamination.

2.2. EV isolation

A431 cells were seeded in T175 flasks and cultured for 24 h, after which medium was replaced by serum-free Opti-MEM (Thermo Fisher Scientific) supplemented with 100 U/mL penicillin, 100 U/mL streptomycin. After 48 h, EVs were isolated using a recently described size-exclusion chromatography method [25]. Conditioned medium (CM) was centrifuged for 10 min at 2000 × *g* at 4 °C. Subsequently, CM was vacuum filtered using 0.45 μm bottle top filters (Thermo Fisher Scientific) and concentrated to ≤ 5 mL using 100 kD MWCO Amicon Ultra-15 Centrifugal Filter Units (Millipore) at 4 °C. Concentrated CM was loaded onto a HiPrep 16/60 Sephacryl S-400 HR gel filtration column (GE Healthcare Life Sciences), equilibrated with phosphate-buffered saline (PBS) and connected to an ÄKTA Start chromatography system (GE Healthcare), both maintained at 4 °C. EVs were separated from non-vesicular material using PBS as eluent. EV-containing fractions (as determined by UV absorbance at 280 nm) were pooled, filtered through a 0.45 μm filter and concentrated using 100 kD MWCO Amicon Ultra-15 Centrifugal Filter Units at 4 °C.

2.3. Western blot analysis

Cells or EVs were lysed in RIPA buffer (Alfa Aesar) supplemented with Protease Inhibitor Cocktail (Sigma-Aldrich). Lysates were centrifuged at 12000 × *g* for 15 min at 4 °C to remove insoluble material, after which protein concentrations were determined using a MicroBCA Protein Assay using bovine serum albumin (BSA) as a standard according to the manufacturer's instructions. Lysates were mixed with sample buffer containing dithiothreitol (or without for CD63 analysis), heated to 95 °C for 10 min and subjected to electrophoresis on 4–12% Bis-Tris polyacrylamide gels (Thermo Scientific). Proteins were blotted on Immobilon-FL polyvinylidene difluoride membranes (Millipore), after which membranes were blocked with 50% v/v Odyssey Blocking Buffer (LI-COR Biosciences) in Tris-buffered saline (TBS). Subsequently, membranes were probed with mouse anti-ALIX (1:1000, clone 3A9, Abcam), mouse anti-CD63 (1:600, clone MEM-259, Abcam), mouse anti-actin (1:1000, clone JLA20, Millipore), rabbit anti-calnexin (1:1000, clone N3C2, Genetex), rabbit anti-CLTC (1:1000, polyclonal, Abcam), rabbit anti-CAV1 (1:6000, clone EPR15554, Abcam), rabbit anti-CDC42 (1:6000, EPR15620, Abcam), rabbit anti-flotillin-1 (1:6000, EPR6041, Millipore), rabbit anti-ARF6 (1:1000, EPR8357, Abcam), mouse anti-RhoA (1:1000, 1B12, Abnova), mouse anti-Rac1 (1:1000, 23A8, Millipore), rabbit anti-PAK1 (1:1000, polyclonal, Abcam) or rabbit anti-ANKFY1 (1:1000, polyclonal, Abgent) antibodies in 50% v/v Odyssey Blocking Buffer in TBS with 0.1% Tween20 (TBS-T). Secondary antibodies included Alexa Fluor 680 goat anti-mouse, Alexa Fluor 680 goat anti-rabbit (Thermo Fisher Scientific) and IRDye 800 donkey anti-mouse (LI-COR Biosciences) and were applied at a 1:10000 dilution. Protein bands were visualized on an Odyssey Infrared Imager (LI-COR Biosciences) at 700 and 800 nm.

2.4. Nanoparticle tracking analysis

EV size distribution was determined with a Nanosight NS500 nanoparticle analyser (Malvern Instruments) equipped with a 405 nm laser. A camera level of 15–16 and automatic functions for all post-acquisition settings except for the detection threshold were used. This was fixed at 6. Using the script control function, three 30 s videos for each sample were recorded. Analysis was performed with NTA 3.1 software.

2.5. Transmission electron microscopy

EVs were adsorbed to carbon-coated formvar grids for 15 min at RT. Unbound EVs were removed by washing with PBS. Grids were fixated in 2% paraformaldehyde, 0.2% glutaraldehyde in PBS for 30 min at RT, counterstained with uranyl-oxalate and embedded in a mixture of 1.8% methyl cellulose and 0.4% uranyl acetate at 4 °C. Imaging was done on a Jeol 1010 microscope (Jeol).

2.6. EV labelling and purification

EVs were labeled with 5×10^{-6} M PKH67 (Sigma-Aldrich) according to the manufacturer's instructions. For removal of unincorporated label, XK-16/20 column (GE Healthcare) was packed with sepharose CL-4B (Sigma-Aldrich) according to the manufacturer's instructions. Column was connected to a refrigerated ÄKTA Start chromatography system, equilibrated with PBS, and EV/dye mixtures were injected. Pooled EV fractions were concentrated using 100 kD MWCO Amicon Ultra-15 Centrifugal Filter Units at 4 °C.

2.7. Drug treatments

Cells were preincubated with inhibitors before EV addition. For heparin (Sigma-Aldrich), cytochalasin D (Sigma-Aldrich), chlorpromazine (Sigma-Aldrich), genistein (Sigma-Aldrich), EIPA (Santa Cruz Biotechnology) and wortmannin (Sigma-Aldrich), cells were pretreated for 30 min. For simvastatin (Sigma-Aldrich), cells were pretreated for 20 h. Inhibitors were present throughout experiments.

2.8. Transfections

HeLa cells were seeded at a density of 50000 cells/well in 6-well plates. After 24 h, cells were transfected with siRNA (sequences are listed in Table 1) using Lipofectamine RNAiMax (Life Technologies) according to the manufacturer's instructions. Transfection was repeated after an additional 24 h. At 6 h after the second transfection, medium was replaced and cells were incubated for 24 h and reseeded for EV internalization assays, or for 48 h for Western blot analysis.

2.9. EV internalization assays

For flow cytometric analysis, HeLa cells were seeded at a density of 15000 cells/well in 24-well plates. After 24 h, cells were pretreated with inhibitors as described above and then incubated with PKH67-