Topic Introduction

Strategies for Isolation of Exosomes

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Exosomes are tiny vesicles (diameter 30–150 nm) secreted by cells in culture and found in all body fluids. These vesicles, loaded with unique RNA and protein cargos, have many biological functions, of which only a small fraction is currently understood—for example, they participate in cell-to-cell communication and signaling within the human body. The spectrum of current scientific interest in exosomes is wide and ranges from understanding their functions and pathways to using them in diagnostics, as biomarkers, and in the development of therapeutics. Here we provide an overview of different strategies for isolation of exosomes from cell-culture media and body fluids.

INTRODUCTION

Intercellular communication is an extremely important process that takes place within all multicellular organisms. It can be mediated through direct cell-to-cell contact or by transfer of secreted small molecules. A few years ago, a third mechanism for intercellular communication was discovered, which depends on the production of extracellular vesicles. Although the release of apoptotic bodies from cells during the process of cell death had been observed previously (Hristov et al. 2004), the discovery that healthy cells also constantly release various types of vesicles into the extracellular space was a huge breakthrough.

The Properties of Exosomes

The term "exosome" was first coined by Trams and colleagues in 1981 for vesicles secreted by cell lines with ectoenzyme activity (Trams et al. 1981). Later, this nomenclature was adopted for the vesicles of diameter ~30–150 nm that are released during differentiation of reticulocytes as a consequence of multivesicular endosome fusion with the plasma membrane (Harding et al. 1984; Pan et al. 1985). Shortly thereafter, exosomes were found to be released by B lymphocytes and dendritic cells through a similar route (Raposo et al. 1996; Zitvogel et al. 1998). In subsequent years, many additional cell types of both hematopoietic and nonhematopoietic origin, such as cytotoxic T cells, platelets, mast cells, neurons, oligodendrocytes, Schwann cells, and intestinal epithelial cells, have been shown to release exosomes (Simons and Raposo 2009; Théry et al. 2009). Furthermore, exosomes have been found in many body fluids, including blood (Caby et al. 2005), semen (Aalberts et al. 2012), urine (Pisitkun et al. 2004), saliva (Ogawa et al. 2011), breast milk (Admyre et al. 2007), ascites fluid (Andre et al. 2002), amniotic fluid (Asea et al. 2008), cerebrospinal fluid (Vella et al. 2007), and bile (Masyuk et al. 2010).

Owing to their endosomal origin, all exosomes contain proteins participating in membrane transport and fusion (GTPases, annexins, flotillin), tetraspanins (CD9, CD63, CD81, CD82), heat-shock

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proteins (Hsc70, Hsp90), proteins involved in biogenesis of multivesicular bodies (Alix, TSG101), and lipid-related proteins and phospholipases (Conde-Vancells et al. 2008; Subra et al. 2010). More than 4500 different proteins have been identified in association with exosomes, usually by mass spectrometry, presumably serving as cargo for intercellular communication (Mathivanan and Simpson 2009). In addition to proteins, exosomes are enriched in certain raft-associated lipids, such as cholesterol (primarily B lymphocytes), ceramide (implicated in the differentiation of exosomes from lysosomes), other sphingolipids, and phosphoglycerides with long and saturated fatty-acyl chains (Wubbolts et al. 2003; Subra et al. 2007; Trajkovic et al. 2008).

It has been shown that exosomes also contain RNA, in particular miRNA (more than 100 sequences) and mRNA (more than 1300 sequences), some of which could be translated into proteins by target cells (Ratajczak et al. 2006; Valadi et al. 2007). Later studies reported on the RNA contents of vesicles isolated from other cell cultures (Skog et al. 2008) and from body fluids (Hunter et al. 2008; Rabinowits et al. 2009; Michael et al. 2010). Recent deep-sequencing experiments have shown that exosomes actually contain a very diverse RNA cargo, including hundreds of noncoding RNAs (snRNA, snoRNA, scaRNA, piRNA), tRNA, and rRNA (Schageman et al. 2013; Bellingham et al. 2012; Nolte-'t-Hoen et al. 2012). Most of the RNA cargo is 20–200 nucleotides in length, including full-length molecules such as miRNA and tRNA, and fragments of long RNA (mRNA, rRNA)—although some molecules are present in full-length forms (Zeringer et al. 2013).

The Functions of Exosomes

Depending on the cell or tissue of origin, many different roles and functions have been attributed to exosomes—to name a few: eradication of obsolete molecules, facilitation of the immune response (Théry et al. 2009), antigen presentation, programmed cell death, angiogenesis, inflammation, coagulation, dissemination of oncogenes from tumor cells, and spread of pathogens such as prions and viruses from one cell to another (Vella et al. 2007). Most importantly, exosomes have been shown to deliver macromolecular messages (RNA and protein), enabling cell-to-cell communication and signaling (Vlassov et al. 2012). Interest toward exosomes—ranging from their function in the body to more practical applications, such as their use in diagnostics and biomarker development (based on analysis of their RNA and protein content) and therapeutics—has grown exponentially in the past five years.

EXOSOME PURIFICATION METHODS

Exosome Isolation by Ultracentrifugation

The accepted protocol for isolation of exosomes includes ultracentrifugation, often in combination with sucrose density gradients or sucrose cushions to float the relatively low-density exosomes (Théry et al. 2006). The ultracentrifugation approach has several drawbacks: The method is highly labor-intensive and time-consuming (up to 2 days per preparation, for a protocol with sucrose gradients); one cannot process more than six samples at a time (owing to limitations of the design of ultracentrifuge rotors); the method requires a large amount of starting material; exosome yields are typically low; and extensive training of personnel is needed. However, sequential centrifugations, when combined with sucrose gradient ultracentrifugation, can produce exosome preparations of high purity, and this method for exosome isolation has been successfully used by many laboratories over the past decade.

Size-Based Isolation of Exosomes

Isolation of exosomes based on size, using alternatives to the ultracentrifugation routes, is an obvious option. Cheruvanky et al. (2007) reported the successful purification of exosomes by using ultrafiltra-

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tion procedures that are less time-consuming than ultracentrifugation and do not require the use of special equipment. Similarly, Bioo Scientific launched a kit termed ExoMir that, essentially, removes all cells, platelets, and cellular debris on one microfilter and captures all vesicles of diameter larger than 30 nm on the second microfilter, using positive pressure to drive the fluid. For this process, the exosomes themselves are not reclaimed—but their RNA content is directly extracted off the material caught on the second microfilter and can then be used for PCR analysis (for details, see www.BiooScientific.com). HPLC (high-performance liquid chromatography)-based protocols could potentially allow the procurement of highly pure exosomes, although these processes require dedicated equipment and are not trivial to scale up (Lai et al. 2010). The complication is that both body fluids and cell-culture media contain a large number of nanoparticles (some nonvesicular) in the same size range as exosomes. For example, Wang et al. (2010) found that a large number of miRNAs are contained within extracellular protein complexes rather than exosomes (the biological roles for these are yet to be established). As a consequence, the above methods are best described as allowing one to obtain exosome-enriched samples, rather than pure exosomes.

Exosome Precipitation

Volume-excluding polymers such as polyethylene glycols (PEGs) are routinely used for precipitation of viruses and other small particles (Yamamoto et al. 1970; Adams 1973; Lewis and Metcalf 1988). This principle, or perhaps differential solubility in alternative solvents, could be applied to precipitate exosomes from experimental samples. The precipitate can be isolated using either low-speed centrifugation or filtration. System Biosciences offers a proprietary reagent named ExoQuick, which can be added to conditioned cell media, serum or urine, that precipitates the exosomes (www.systembio.com). Life Technologies launched five Total Exosome Isolation reagents, enabling fast recovery of the entire exosome population from various sample types (www.lifetechnologies.com/exosomes). The reagents share the same core component but are optimized specifically for cell-culture media, serum, plasma, urine, and other body fluids (saliva, milk, cerebrospinal fluid, ascitic fluid, and amniotic fluid). By tying up water molecules, the reagents force less-soluble components such as exosomes out of solution, and the exosomes can be conveniently collected by low-speed centrifugation.

Affinity-Based Capture of Exosomes

In theory, a superior alternative for specific isolation of exosomes should be affinity purification with antibodies to CD63, CD81, CD82, CD9, Alix, annexin, EpCAM, and Rab5. These could be used by themselves or, potentially, in combination. For this application, the antibodies could be immobilized on a variety of media, including magnetic beads, chromatography matrices, plates, and microfluidic devices (Théry et al. 2006; Chen et al. 2010). HansaBioMed (www.hansabiomed.eu) and Life Technologies (www.lifetechnologies.com/exosomes) offer an array of products featuring antibodies against CD63, CD81, or CD9 for exosome capture and characterization. As with any young field, it remains to be confirmed how well these systems work and, for researchers wanting a diverse exosome population, which of these proteins is (are) the best and most-robust exosomal tag(s) for their needs. In the same vein as antibodies, other affinity-capture methods could be used, such as lectins, which will bind to specific saccharide residues on the exosome surface. This strategy has been proposed by Aethlon Medical (www.aethlonmedical.com), using a proprietary lectin that targets mannose residues. The convenient feature of this procedure is easy elution of the captured exosomes by free α-methyl-mannoside. However, this approach is not specific to exosomes as a number of cells contain mannose on their surface, and it has yet to be proven whether all exosome types can be captured this way. Multiple types of lectins are available, and these can be carefully investigated to select the best options. Vn peptides were also recently reported to be capable of efficiently binding to exosomes, and the commercial product based on Vn96 peptide is now available at New England Peptide (www.newenglandpeptide.com). As in the case of lectins, little is known about the practicability of this approach presently, but the methodology is very interesting and definitely worth investigating.

VESICLES WITH A BIG FUTURE

In the past few years, there has been an exponential increase in the number of studies aiming to understand the biology of exosomes, as well as other nanovesicles and microvesicles. Every day, we gain more insight and knowledge about the mechanisms of their formation, secretion, pathways in vivo, and internalization into recipient cells and the biological roles of their protein and nucleic acid cargos. Despite being a young area of research, it is clear that the utility of these novel vesicles goes beyond basic research but extends into applications in the fields of diagnostics and therapeutics. Crucial to further our understanding of exosomes is the continuing development of reagents, tools, and protocols for their isolation and characterization and for analysis of their RNA and protein contents. The above-described strategies for exosome isolation provide a good starting point; however, it is clear that more advanced tools are required to study exosomes and other extracellular vesicles.

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