Methods 56 (2012) 293–304

Contents lists available at SciVerse ScienceDirect

Methods

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

Comparison of ultracentrifugation, density gradient separation, and immunoaffinity capture methods for isolating human colon cancer cell line LIM1863-derived exosomes

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ARTICLE INFO

Article history:
Available online 21 January 2012

Keywords:
Exosome
EpCAM
OptiPrep™
Immunoaffinity capture
Density
Centrifugation
LIM1863
Colon cancer
Microvesicles

ABSTRACT

Exosomes are 40–100 nm extracellular vesicles that are released from a multitude of cell types, and perform diverse cellular functions including intercellular communication, antigen presentation, and transfer of oncogenic proteins as well as mRNA and miRNA. Exosomes have been purified from biological fluids and *in vitro* cell cultures using a variety of strategies and techniques. However, all preparations invariably contain varying proportions of other membranous vesicles that co-purify with exosomes such as shed microvesicles and apoptotic blebs. Using the colorectal cancer cell line LIM1863 as a cell model, in this study we performed a comprehensive evaluation of current methods used for exosome isolation including ultracentrifugation (UC-Exos), OptiPrep™ density-based separation (DG-Exos), and immunoaffinity capture using anti-EpCAM coated magnetic beads (IAC-Exos). Notably, all isolations contained 40–100 nm vesicles, and were positive for exosome markers (Alix, TSG101, HSP70) based on electron microscopy and Western blotting. We employed a proteomic approach to profile the protein composition of exosomes, and label-free spectral counting to evaluate the effectiveness of each method. Based on the number of MS/MS spectra identified for exosome markers and proteins associated with their biogenesis, trafficking, and release, we found IAC-Exos to be the most effective method to isolate exosomes. For example, Alix, TSG101, CD9 and CD81 were significantly higher (at least 2-fold) in IAC-Exos, compared to UG-Exos and DG-Exos. Application of immunoaffinity capture has enabled the identification of proteins including the ESCRT-III component VPS32C/CHMP4C, and the SNARE synaptobrevin 2 (VAMP2) in exosomes for the first time. Additionally, several cancer-related proteins were identified in IAC-Exos including various ephrins (EFNB1, EFNB2) and Eph receptors (EPHA2–8, EPHB1–4), and components involved in Wnt (CTNNB1, TNIK) and Ras (CRK, GRB2) signalling.

1. Introduction

Exosomes are a discrete population of small (40–100 nm diameter) membranous vesicles that are released into the extracellular space from multivesicular bodies (MVBs) by most cell types [1,2]. Typically, the monitoring of exosome isolation has been based upon their size, morphology, flotation density and the presence of marker proteins such as Alix, TSG101, HSP70 and CD9 [1]. Recently, it has been shown that exosomes are also present in body fluids such as synovial fluid [3], saliva [4], urine [5], semen [6], breast milk [7] and, importantly, blood [8]. Originally, exosomes were implicated in the mechanism for removal of cell surface molecules in reticulocytes [9–11] followed shortly thereafter as possible vehicles for antigen presentation [12,13] and immune suppression in cancer [14,15]. More recently, exosomes have

Abbreviations: CM, culture medium; CCM, concentrated culture medium; EM, electron microscopy; EpCAM, epithelial cell adhesion molecule; ESCRT, endosomal sorting complex required for transport; FCS, foetal calf serum; HSP, heat shock protein; ILV, intralumenal vesicle; IMP, integral membrane protein; ITS, insulin–transferrin–selenium; LDH, lactate dehydrogenase; MACS, magnetic activated cell sorting; MVB, multivesicular body; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PDCD6IP/Alix, programmed cell death 6 interacting protein; SMART, simple modular architecture research tool; SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor; TEM, tetraspanin enriched microdomains; TMHMM, transmembrane hidden Markov model; TSG101, tumour susceptibility gene 101.

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doi:10.1016/j.ymeth.2012.01.002

gained much attention for their important role in intercellular communication [16–18]. For example, exosomes have been reported to provide a mechanism for generating soluble cytokine receptors via protease-dependent [19] or protease-independent receptor ectodomain cleavage [20]. In a seminal study, cancer cell derived-microvesicles containing oncogenic proteins (e.g., the truncated, oncogenic form of EGFRv111) – referred to as 'oncosomes' – have been shown to traverse the tumour microenvironment and be taken up by recipient EGFv111 receptor null cells leading to transfer of oncogenic activity [21]. Additionally, cancer-derived exosomes have been reported to contain tumour progression related proteins such as L1CAM, CD24, ADAM10, and EMMPRIN [22] and amphiregulin [23]. Moreover, they have also been shown to initiate proangiogenic signalling cascades in melanoma cells [24]. In addition to proteins, exosomes have also been shown to be carriers of endogenous mRNAs and miRNAs [25,26] and lipid mediators [27], which can modulate the translational activity of recipient cells. Clinically, there is growing interest in the potential use of exosomes as disease biomarkers (e.g., miRNA signatures from disease-derived exosomes circulating in blood [28]), vaccine candidates for tumour immunotherapy [29] (for reviews, see [30,31]), gene delivery vehicles (e.g., siRNA carriers [32]; for a review/commentary, see [33,34]) and as mediators of myocardial ischaemia/reperfusion injury [35]. Despite recent advances in our understanding of exosome biology, much of this information has been obtained from impure exosome preparations, which have confounded interpretation of findings. For example, it is well known that eukaryotic cells release many membranous particle types into the microenvironment, these include exosomes, exosome-like microparticles, shedding microvesicles (SMVs), apoptotic blebs (ABs) [18] and the recently described 'gesicles' [36]. Hence, there is an urgent need to better define exosome preparations so that information obtained at both protein and RNA levels can be appropriately interpreted with respect to unambiguous biological function. Likewise, it is important to accurately define homogeneous exosome populations before embarking on large-scale production for the purpose of detailed biochemical analyses and/or preparation of clinical-grade reagents.

It is well recognised that cell culture media contain, in addition to cell detritus, several types of released membranous vesicles [18]. Thus, it is important to work with as pure a sample as possible, especially when undertaking functional exosome studies. Current strategies for purifying and characterising exosomes from cell culture medium or body fluids differ significantly. In the original and widely-used method for purifying exosomes from culture media [37], differential ultracentrifugation was employed to first remove intact cells and bulky cell debris by low *g* force centrifugation (e.g., 500*g*, 2000*g*) followed by high *g* force (e.g., 100,000*g*) to sediment exosomes. In some strategies, the initial low speed centrifugation step(s) has been replaced by 0.1 μm [38] or 0.22 μm [39] filtration or inclusion of an intermediate *g* force centrifugation step (e.g., 60,000*g*) to remove shed microvesicles (500–2000 nm diameter) [40]. In order to purify exosomes from viscous body fluids such as plasma or malignant ascites using differential centrifugation, it is necessary to include a dilution step to reduce the viscosity, and to increase both the centrifugal force and centrifugation time [41]. One possible drawback of using differential centrifugation for isolating exosomes is co-sedimentation of protein aggregates and co-purifying non-specifically bound proteins. As well as confounding the interpretation of MS-based protein identifications, it has been demonstrated that protein aggregates are ~10,000 times more immunogenic than the corresponding soluble form because of preferential capture by antigen presenting cells [42]. One way of separating large protein aggregates from exosomes is by ultracentrifugation using a linear sucrose gradient to exploit their different flotation densities [43]; typically, exosomes have a buoyant floatation density of 1.08–1.22 g/mL on sucrose gradients [37]. For the preparation of GMP-grade exosomes for clinical purposes, a combination of ultrafiltration, ultracentrifugation and a 30% sucrose/deuterium (D_2O) (98%) cushion (1.21 g/mL) has been recently described [44]. Interestingly, sucrose gradients have been shown to be inefficient in separating exosomes from HIV-1 particles due to similarities in their size/diameter and buoyant density. To overcome this problem, Cantin and colleagues describe the use of iodixanol (OptiPrep™) 6–18% gradients to separate HIV-1 particles and apoptotic vesicles from exosomes [45]. A rapid and simple method for isolating exosomes from culture media as well as body fluids is by immunoisolation employing magnetic beads. Exosome pull-down based on immunoaffinity can be a powerful isolation tool provided a specific exosomal cell surface protein can be identified that discriminates an exosome of interest from other membranous particles present in the biological matrix (for a list of exosomal protein markers see [43]). Immunoisolation of exosomes has been performed for antigen presenting cells [46], as well as HER2-positive exosomes from breast adenocarcinoma cell lines and ovarian cancer patient-derived ascites [47]. In addition, A33-positive exosomes released from colon carcinoma cancer cells [48], and EpCAM-positive exosomes from the sera of lung cancer [8] and ovarian cancer [28] patients have been obtained.

In this study, the culture medium of LIM1863 colorectal carcinoma cells was used to compare the morphological and proteomic profiles of exosomes purified by three different isolation strategies: ultracentrifugation (UC-Exos), density gradient centrifugation using OptiPrep™ (DG-Exos), and immunoisolation using EpCAM antibodies coupled to magnetic beads (IAC-Exos). To assess the three purification strategies we monitored the enrichment of several protein classes that have been inextricably associated with exosome biogenesis and/or function – endosomal sorting complex required for transport (ESCRT)-complex and their associated proteins, Rab GTPases, tetraspanins, proteins implicated in intracellular trafficking, as well as proteins that may be involved in exosome internalisation in a recipient cell. To enable this comparative enrichment assessment, we employed a proteomic label-free peptide spectral count strategy that entails summating the number of significant peptide MS/MS spectra for each individual protein, and normalising them with respect to the total number of spectra identified in that particular sample. The normalised ratios can then be compared between samples to estimate enrichment. Our findings indicate that immunoaffinity capture was the most efficient technique to enrich for exosomes compared to differential centrifugation and density gradient isolation methods.

2. Material and methods

2.1. Cell culture and preparation of concentrated culture medium (CCM)

Human colon carcinoma LIM1863 cells [49] were cultured in RPMI-1640 medium (Invitrogen, Carlsbad, CA) containing 5% FCS, α-thioglycerol (10 μM), insulin (25 units/L), hydrocortisone (1 mg/L), with 10% CO_2 at 37 °C. LIM1863 cells (~2×10^9 cells) were washed four times with 30 mL of RPMI-1640 media and cultured for 24 h in 750 mL serum-free RPMI-media supplemented with 0.6% insulin–transferrin–selenium (ITS) solution from Invitrogen. Approximately 750 mL of culture medium (CM) was collected and centrifuged at 4 °C (480*g* for 5 min followed by 2000*g* for 10 min) to remove intact cells and cell debris. CM was filtered using a VacuCap® 60 filter unit fitted with a 0.1 μm Supor® membrane (Pall Life Sciences, Port Washington, NY) and then concentrated to 1.5 mL using an Amicon® Ultra-15, Ultracel centrifugal filter device with a 5 K nominal molecular weight limit (NMWL)