Methods xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Methods

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

Methods of isolating extracellular vesicles impact down-stream analyses of their cargoes

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ARTICLE INFO

Article history:
Received 30 December 2014
Received in revised form 25 February 2015
Accepted 26 February 2015
Available online xxxx

Keywords:
Exosomes
Isolation
Ultracentrifugation
Chromatography
Immunoaffinity

ABSTRACT

Viable tumor cells actively release vesicles into the peripheral circulation and other biologic fluids, which exhibit proteins and RNAs characteristic of that cell. Our group demonstrated the presence of these extracellular vesicles of tumor origin within the peripheral circulation of cancer patients and proposed their utility for diagnosing the presence of tumors and monitoring their response to therapy in the 1970s. However, it has only been in the past 10 years that these vesicles have garnered interest based on the recognition that they serve as essential vehicles for intercellular communication, are key determinants of the immunosuppressive microenvironment observed in cancer and provide stability to tumor-derived components that can serve as diagnostic biomarkers. To date, the clinical utility of extracellular vesicles has been hampered by issues with nomenclature and methods of isolation. The term "exosomes" was introduced in 1981 to denote any nanometer-sized vesicles released outside the cell and to differentiate them from intracellular vesicles. Based on this original definition, we use "exosomes" as synonymous with "extracellular vesicles." While our original studies used ultracentrifugation to isolate these vesicles, we immediately became aware of the significant impact of the isolation method on the number, type, content and integrity of the vesicles isolated. In this review, we discuss and compare the most commonly utilized methods for purifying exosomes for post-isolation analyses. The exosomes derived from these approaches have been assessed for quantity and quality of specific RNA populations and specific marker proteins. These results suggest that, while each method purifies exosomal material, there are pros and cons of each and there are critical issues linked with centrifugation-based methods, including co-isolation of non-exosomal materials, damage to the vesicle's membrane structure and non-standardized parameters leading to qualitative and quantitative variability. The down-stream analyses of these resulting varying exosomes can yield misleading results and conclusions.

1. Introduction

Our group previously demonstrated the release of 50–200 nm membranous vesicles by tumor cells into their extracellular environment [1], which have been referred to as exosomes, microvesicles or extracellular vesicles depending on specific characteristics, including size, composition and biogenesis pathway. Since our original demonstration, the release of vesicles has since been demonstrated multiple cell types and systems. In cancer patients, these nanometer-sized vesicles released by tumor cells accumulate in biologic fluids, including blood, urine, ascites, and pleural fluids [2]. These cell-derived vesicles exhibit an array of proteins, lipids and nucleic acids derived from the originating tumor. These tumor-derived exosomes not only represent a central mediator of the tumor microenvironment, but their presence in the peripheral circulation may serve as a surrogate for tumor biopsies, enabling non-invasive diagnosis and real-time disease monitoring [3].

Although the release of exosomes occurs in other types of cells under specific physiological conditions, the increased release of vesicles and their accumulation appear to be important in the malignant transformation process. Recently, circulating vesicles from normal individuals, patients with benign ovarian disease and patients with ovarian cancer have been investigated using the Nanoparticle Tracking Analysis system (Nanosight) [4]. The presence of circulating vesicular materials was demonstrated in all individuals; however, ovarian cancer patients exhibit approximately 3–4-fold more vesicular material. In these cancer patients, the size range of these vesicles was between 50 and 250 nm, with the major peak at 98–99 nm (Fig. 1). The identification of specific tumor-derived vesicles using fluorescent-label

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http://dx.doi.org/10.1016/j.ymeth.2015.02.019
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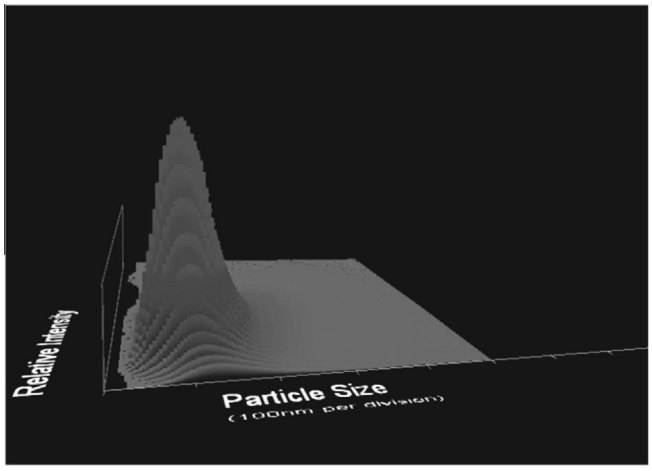

Particle Size / Relative Intensity 3D plot

Fig. 1. Distribution of total vesicles in serum of a patient with stage III adenocarcinoma of the ovary. Serum was centrifuged at 400×*g* for 10 min and this resulting supernatant was centrifuged at 15,000×*g* for 15 min. The supernatant was then diluted 1:4 in PBS and analyzed using a Nanosight NS300 in light scatter mode. The Nanoparticle tracking analysis software defined the number and size range of the vesicles within the sample, plotting the particle size versus relative intensity versus number.

antibody against tumor markers, indicate that, even in advanced stage patients, only approximately 10% of the total exosomes are tumor-derived; the remainder of the increased vesicles are likely the result of the host response to the tumor. Some of the enhanced exosome numbers derived from the host's response to the tumor appears to be derived from immune cells (Fig. 2).

These circulating vesicles have been identified by various terms, including high molecular weight complexes, membrane fragments, exosomes, microvesicles, microparticles, and extracellular vesicles, as well as by functional names. The term "exosome" was coined in 1981 for "exfoliated membrane vesicles with 5′-nucleotidase activity" [5]. This term, "exosome," originated from the discovery of neoplastic cell line-derived exfoliated vesicles, which mirrored the 5′-nucleotidase activity of the parent cells [5]. In ovarian cancer patients, these tumor-derived exosomes were found to express molecular markers that were linked with tumor plasma membranes, including placental type alkaline phosphatase and mdr-1 [6–8]; however, proteins not generally associated with plasma membranes, such as p53, GRP78 and nucleophosmin, have also been identified with these circulating vesicles [9,10]. These findings emphasize the aberrant sorting of components into exosomes in cancer and may differentiate cancer vesicles from their normal counterparts.

Several years after these early characterizations of exosomes from tumor cells, two groups studying maturation in cultured reticulocytes (sheep [11] and rat [12]) examined vesicles released via the canonical pathway upon multi-vesicular endosome fusion with the cell surface. The vesicles were isolated by ultracentrifugation and the pelleted vesicles were found to contain the transferrin receptor that was also found in native reticulocytes [13]. These reports proposed that this represented a mechanism for the elimination of certain cellular components as the reticulocytes matured and differentiated. These investigators "re-defined" these cell-derived vesicles as "exosomes" to differentiate them from "endosomes." The disparate natures of these studies are reflected in the various names that were proposed and which are still used to identify the cell surface-released and endocytic vesicles of different origins. It is of note that these reticulocyte studies (11–13) were exclusively *in vitro* and based on normal cell types, undergoing a specific differentiation pathway. Likewise, the characteristics currently used to define "microvesicles" were derived from studies on normal B cells *in vitro* and may not translate to vesicles derived from other cell types, particularly tumor cells [14]. While many investigators use these restrictive definitions for cell-derived vesicles without understanding their origin, significant overlap exists between structures identified as "exosomes" and "microvesicles," in terms of size, markers, cargoes and function, particularly in the context of transformed cells. Within the circulation, it may not be possible to differentiate 50–100 nm "exosomes" from 50 to 200 nm "microvesicles." Investigators have attempted to define exosomes versus microvesicles, based on size (30–100-nm lipid bilayer vesicles), density (1.12–1.19 g/ml) and expression of specific biomarkers (including tetraspanins) [15].

Using the Nanosight in fluorescent mode to analyze culture-derived tumor vesicles, we have demonstrated the presence of "exosome specific" markers on vesicles over the entire 50–

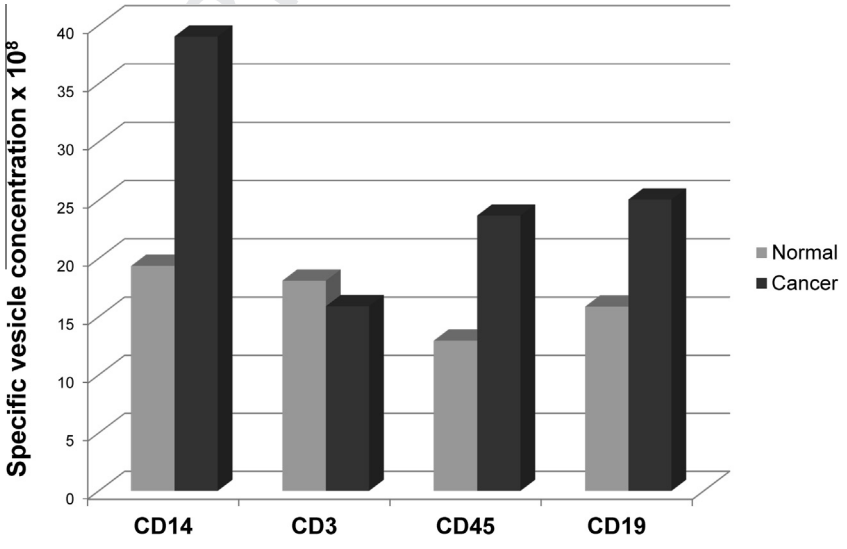

Fig. 2. Total exosomes were isolated from the serum of a normal female control and an ovarian cancer patient by size exclusion chromatography. Specific exosome populations from immune cells were isolated from each serum by immunoaffinity capture using immobilized antibodies against CD14, CD3, CD45, and CD19. The number of exosomes obtained with each antibody was determined using the Nanosight NS300 in light scatter mode.

Please cite this article in press as: D.D. Taylor, S. Shah, Methods (2015), http://dx.doi.org/10.1016/j.ymeth.2015.02.019