Journal of Immunological Methods 411 (2014) 55–65

Contents lists available at ScienceDirect

Journal of Immunological Methods

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

Research paper

Isolation of biologically-active exosomes from human plasma

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ARTICLE INFO

Article history:
Received 3 May 2014
Received in revised form 8 June 2014
Accepted 9 June 2014
Available online 18 June 2014

Keywords:
Exosome isolation
Human plasma
Size-exclusion chromatography
Immunological studies
Exosome characteristics

ABSTRACT

Effects of exosomes present in human plasma on immune cells have not been examined in detail. Immunological studies with plasma-derived exosomes require their isolation by procedures involving ultracentrifugation. These procedures were largely developed using supernatants of cultured cells. To test biologic activities of plasma-derived exosomes, methods are necessary that ensure adequate recovery of exosome fractions free of contaminating larger vesicles, cell fragments and protein/nucleic acid aggregates. Here, an optimized method for exosome isolation from human plasma/serum specimens of normal controls (NC) or cancer patients and its advantages and pitfalls are described. To remove undesirable plasma-contaminating components, ultrafiltration of differentially-centrifuged plasma/serum followed by size-exclusion chromatography prior to ultracentrifugation facilitated the removal of contaminants. Plasma or serum was equally acceptable as a source of exosomes based on the recovered protein levels (in μg protein/mL plasma) and TEM image quality. Centrifugation on sucrose density gradients led to large exosome losses. Fresh plasma was the best source of morphologically-intact exosomes, while the use of frozen/thawed plasma decreased exosome purity but not their biologic activity. Treatments of frozen plasma with DNAse, RNAse or hyaluronidase did not improve exosome purity and are not recommended. Cancer patients' plasma consistently yielded more isolated exosomes than did NCs' plasma. Cancer patients' exosomes also mediated higher immune suppression as evidenced by decreased CD69 expression on responder CD4+ T effector cells. Thus, the described procedure yields biologically-active, morphologically-intact exosomes that have reasonably good purity without large protein losses and can be used for immunological, biomarker and other studies.

1. Introduction

Exosomes are virus-sized vesicles, ranging from 20 to 100 nm in size, and enveloped by a phospholipid membrane (Thery et al., 2002). Formed within the endocytic compartments, exosomes are released into the extracellular space via fusion of multivesicular bodies with the cell surface membrane (Thery et al., 2002). They are produced by normal as well as malignant cells and are present in all human body fluids, including blood (Hawari et al., 2004; Graner et al., 2009), urine (Pisitkun et al., 2004), cerebrospinal fluid (Harrington et al., 2009; Street et al., 2012) and ascites (Runz et al., 2007). Exosomes carry multiple membrane-tethered, biologically-active molecules, and their molecular cargo mimics the surface molecular profile of the mother cell (Skog et al., 2008; Mathivanan et al., 2010). Vesicular content of exosomes includes nucleic acids, enzymes, soluble

Abbreviations: Ab, antibody; AD, active disease; EXO, exosomes; HNSCC, head and neck squamous cell carcinoma; MF, mean fluorescence intensity; NC, normal control; PBS, phosphate buffered saline; Pt, patient; TEM, transmission electron microscopy; TEX, tumor-derived exosomes.

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http://dx.doi.org/10.1016/j.jim.2014.06.007
0022-1759/

factors and a variety of molecules derived from the cytosol of mother cells (Mathivanan and Simpson, 2009). Tumor cells are avid exosome producers, and we have previously shown that these tumor-derived exosomes, in contrast to exosomes produced by normal cells, exert immunosuppressive effects, impairing survival and functions of adaptive and innate immune cells (Taylor et al., 2003; Kim et al., 2005).

The nomenclature adopted for exosomes is based on their size and a mode of release from mother cells, with vesicles ranging in diameter from 30 to 150 nm and formed as intraluminal vesicles by budding into multivesicular endosomes (MVEs) considered to be exosomes. In contrast, larger (200–500 nm) vesicles which bud directly from the plasma membrane are referred to as "microvesicles" (MVs) (Raposo and Stoorvogel, 2013). There is also some evidence that microvesicles might have properties and functions distinct from exosomes (Raposo and Stoorvogel, 2013).

Studies of exosomes have been largely performed using supernatants of cultured cells. This is because in a cell culture, the origin of exosomes can be determined and because relatively simple chemical composition of most culture media facilitates isolation of exosomes devoid of 'contaminating' proteins, lipids and sugars. Human or animal serum used as a supplement for cell cultures is generally ultracentrifuged prior to its use to remove "contaminating" extracellular vesicles. Isolation of exosomes from plasma or other body fluids is more complex for several reasons. First, the source of exosomes present in plasma is unknown, as plasma contains a mix of exosomes derived from many different cells in varying proportions. Second, plasma exosomes are 'coated' with proteins, glycoproteins or glycolipids likely to cause their aggregation and a potential loss upon subsequent centrifugation. Third, separation of exosomes derived from a specific cell type is a considerable problem, as the vast majority of plasma exosomes originate from erythrocytes and platelets, and the desired exosomal fraction may represent only a small fraction of the total (Vlassov et al., 2012; Witwer et al., 2013; Gyorgy et al., 2014; Van der Meel et al., 2014).

Few attempts have been made to optimize exosome isolation from human plasma (Witwer et al., 2013). The most commonly used technique to isolate exosomes relies on their physical properties: because they are virus-size, they can only be isolated by ultracentrifugation and have a characteristic buoyancy of 1.10 to 1.19 g/mL on continuous sucrose density gradients (Thery et al., 2006; Keller et al., 2007). However, human body fluids represent complex mixtures of many components, including vesicles of several different sizes, protein complexes, protein–nucleic acid aggregates and subcellular fragments. Thus, isolation of well-defined exosomal fractions free of "contaminating" vesicular and non-vesicular components from human plasma for biologic studies is a challenging task. The tendency of exosomes to form aggregates of varying sizes results in losses, which further complicate their recovery.

We have previously used size-exclusion chromatography originally described by Taylor et al. (2002) prior to ultracentrifugation to isolate purified exosomes from cell supernatants or human plasma (Taylor et al., 2003; Kim et al., 2005). In this manuscript, we show that application of this methodology following differential centrifugation of human plasma and ultrafiltration results in increased purity and greater recovery of morphologically and functionally intact exosomes. In view of increasing recognition of exosomes as potentially useful biomarkers of disease, our goal was to obtain pure exosomal fractions and to optimize their quantitative recovery from human fresh or banked plasma specimens.

2. Materials and methods

2.1. Peripheral blood specimens

Buffy coats obtained from healthy volunteers were purchased from the Central Blood Bank of Pittsburgh. Under an IRB-approved protocol (IRB #991206) venous blood samples were obtained from cancer patients or normal controls (NC). All subjects signed an informed consent prior to blood draws. Venous blood was collected into tubes with or without added heparin. Heparinized blood was centrifuged on Ficoll-Hypaque gradients (GE Healthcare Bioscience). PBMC were recovered, washed in AIM-V medium (Invitrogen, Grand Island, NY, USA) and were immediately used for experiments. Plasma or serum was harvested and either immediately used or aliquoted into 2 mL vials and banked in liquid N_2 until future use.

2.2. Exosome isolation

Exosomes were isolated from human plasma as previously described (Kim et al., 2005) with the modifications that included differential centrifugation of plasma (1000 × g for 10 min at 4 °C and 10,000 × g for 30 min at 4 °C) followed by ultrafiltration (0.22 μm filter; Millipore, Billicera, MA, USA) and modified size-exclusion chromatography. If high lipid content was present after the low-speed centrifugation (as evident by color), plasma was incubated for 2 h at 4 °C, and the precipitated fat was removed by centrifugation at 1000 × g for 10 min at 4 °C. A 9 mL aliquot of plasma was applied to an A50m column (Bio-Rad Laboratories, Hercules, Ca, USA) packed with Sepharose 2B (Sigma-Aldrich, St. Louis, MO, USA), and the exclusion volume fractions (#2 and #3, each 9 mL) were retained, while fraction #1 was discarded. Following ultracentrifugation (Fig. 1), the exosome pellet was resuspended in PBS and protein concentration was measured. Isolated exosomes were immediately used for experiments or stored at 4 °C. For a long-period storage, exosomes were frozen at −80 °C.

2.3. Plasma specimens

Plasma obtained from cancer patients or NC was used fresh or subjected to one freeze/thaw cycle (rapid freezing at −80 °C for 1 h and slow thawing at 4 °C). In some experiments, after a freeze/thaw cycle and prior to any exosome processing, deoxyribonuclease I (DN25, Sigma, St. Louis, MO, USA) and ribonuclease A (R6513, Sigma) and/or heparinase ((75 Sigma units; H2519) were added. In some experiments, ultrafiltration or size exclusion chromatography was omitted.

2.4. Protein measurements

Aliquots (5–10 μL) of isolated exosomes were dispensed into wells of a 96-well plate, and the assay was performed as recommended by the manufacturer (Pierce BCA Protein Assay Kit, Thermo Scientific, Rockford, lL-61105, USA). Total protein concentrations were determined using a linear standard curve established with bovine serum albumin (BSA).