Chapter 14

Analysis of Extracellular Vesicles in the Tumor Microenvironment

Khalid Al-Nedawi and Jolene Read

Abstract

Extracellular vesicles (ECV) are membrane compartments shed from all types of cells in various physiological and pathological states. In recent years, ECV have gained an increasing interest from the scientific community for their role as an intercellular communicator that plays important roles in modifying the tumor microenvironment. Multiple techniques have been established to collect ECV from conditioned media of cell culture or physiological fluids. The gold standard methodology is differential centrifugation. Although alternative techniques exist to collect ECV, these techniques have not proven suitable as a substitution for the ultracentrifugation procedure.

Key words Exosome, Microvesicle, Ultracentrifugation, Tumor microenvironment

1 Introduction

Extracellular vesicles (ECV) are membrane compartments shed from all types of cells in a variety of physiological and pathological states [1, 2]. There are two main types of extracellular vesicles, namely microvesicles, and exosomes. Microvesicles are generated from the direct outward budding and vesiculation of the plasma membrane into the extracellular milieu [3]. Exosomes, on the other hand, are generated by invagination of the limiting membrane of a multivesicular body (MVB) to form intraluminal vesicles (ILV) [4]. Upon fusion of the MVB with the plasma membrane, ILV are released into the extracellular environment, where they are termed exosomes [4]. In general, microvesicles have average diameters of 200–1000 nm, whereas exosomes range from 50 to 200 nm. The molecular cargo of both classes of ECV comprises proteins, mRNAs [5], miRNAs [6], and even DNAs [7, 8]. In recent years, ECV have garnered much attention for their role in modulating the tumor microenvironment through their role as mediators of intercellular communication [9–12]. Furthermore, ECV have been shown to affect tumor growth by horizontal transfer of oncogenic molecules or tumor

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suppressor proteins among tumor cells and possibly stromal cells [13]. The transfer of such receptors to indolent cells via ECV confers oncogenic and angiogenic phenotypes to target cells [9, 14]. In addition, ECV can transfer mRNA transcripts that can be translated into functional proteins in recipient cells [5], or miRNAs that can regulate translation of target proteins [6].

Further to their role in the tumor microenvironment, ECV are considered a novel source for diagnostic and predictive biomarkers in bodily fluids [15]. Circulating ECV in blood provide a simple, noninvasive platform to look for biomarkers of cancer and other pathologies. Herein, we describe optimal procedures for collection of ECV from cell culture, serum, or plasma samples. In addition, we also describe how to treat cells with ECV and study their effects on a variety of acceptor cells.

2 Materials

2.1 Cell Culture

1. Cell lines. Almost all cell types produce ECV, including primary and immortalized cell lines; therefore ECV can be harvested from the conditioned medium of any cell line of interest.
2. Cell medium. The type of medium and supplements added will be dependent on the cell line used.
3. Tissue culture grade vented flasks (e.g., 75 cm^2).
4. Biosafety cabinet, pipet aids, etc. used for basic mammalian cell culture.
5. Hemocytometer.

2.2 Isolation of ECV

1. Centrifuges. Refrigerated low speed centrifuge, refrigerated high-speed centrifuge, and ultracentrifuge with fixed angle rotor.
2. Centrifuge tubes. 50 mL centrifuge tubes and appropriately sized ultracentrifuge tubes for rotor being used.
3. Phosphate-buffered saline (PBS), pH 7.4.
4. 0.2 μm low-protein-binding filter.

2.3 Quantification of ECV Concentration

1. 2× Protein Sample Buffer (65.8 mM Tris–HCl, pH 6.8, 26.3 % (w/v) glycerol, 2.1 % sodium dodecyl sulfate).
2. DC BioRad Protein Assay or CBQCA Protein Assay.
3. Microplate reader.
4. Nanoparticle Tracking Analysis such as NanoSight LM10.

2.4 Purity Assessment of ECV Preparation

1. Scanning electron microscope (SEM).
2. 0.1 M phosphate buffer, (pH 7.4).
3. 2.5 % glutaraldehyde in 0.1 M phosphate buffer.

4. 0.1 M cacodylate buffer (4.28 g sodium cacodylate, 25 g $CaCl_2$, 2.5 mL 0.2 N hydrochloric acid, diluted to 200 mL with ultrapure water, pH 7.4).
5. 1% osmium tetraoxide.

2.5 Analyzing Transport of ECV-Associated Proteins to Recipient Cells

1. Cell lines.
2. Complete growth medium and serum-free growth medium (components will vary depending on the cell type employed).
3. Tissue culture grade dishes (e.g., 100 mm^2).
4. Biosafety cabinet, pipet aids, etc. used for basic mammalian cell culture.

3 Methods

3.1 Cell Culture

1. Grow the mammalian cells using recommended cell culture protocol for the given cell type. Complete growth medium should contain suitable supplements and bovine serum (BS; *see* **Note 1**). Grow cells in an incubator with a fixed temperature of 37 °C and 5% CO_2.
2. Examine cells daily for growth estimation. For adherent cells, estimate the percentage of confluence. For cells growing in suspension, mix the cell suspension well, remove 100 μL of the cell suspension, and estimate the cell number by hemocytometer or automated cell counter.
3. When cells reach 70% confluence (adherent cells) or approximately 0.5×10^6 cells/mL (suspension cell lines), replace the growth medium with ECV-depleted growth medium (*see* **Note 2**).
4. Grow cells for 24–48 h in the ECV-depleted medium. Then collect the conditioned medium in appropriately sized conical tubes and store on ice.

3.2 Isolation of ECV

Samples should be kept on ice and all centrifugations should be done at 4 °C. The workflow of this procedure is summarized in Fig. 1.

1. Cell debris and large vesicles are removed by passing conditioned medium through a low protein-binding 0.2 μm filter. Centrifuge the filtrate at 300 × *g* for 10 min, collecting the supernatant and discarding the pellet.
2. Transfer the supernatant to a fresh centrifuge tube and centrifuge the sample at 12,000 × *g* for 20 min to remove any remaining cellular debris or large vesicles.
3. Collect the supernatant and transfer sample to fresh ultracentrifuge tubes. Pellet ECV by centrifuging at 100,000 × g for 2 h in ultracentrifuge (*see* **Note 3**). Carefully remove supernatant,

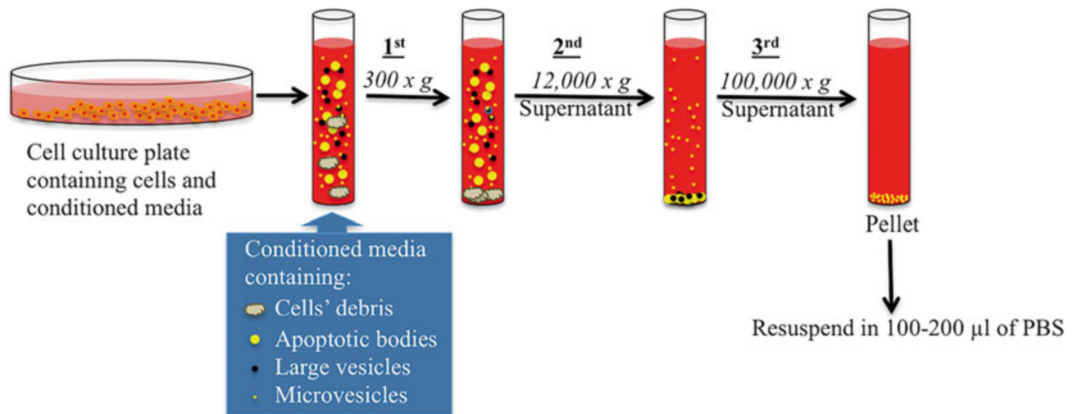

Fig. 1 Collection of extracellular vesicles (ECV) from cells' conditioned media. Cells were grown in suitable growth media supplemented with ECV-depleted FBS. Conditioned media containing cell debris, apoptotic antibodies, large vesicles, and microvesicles were collected and subjected to sequential centrifugation steps to collect ECV. First, conditioned media were subjected to 300 × *g* centrifugation for 5 min to eliminate cell debris. Second, Supernatants from the first step were subjected to 12,000 × *g* centrifugation for 20 min in a high-speed refrigerated centrifuge to remove apoptotic antibodies and large vesicles. Third, the supernatants from the second step were collected and subjected to 100,000 × *g* centrifugation for 2 h at 4 °C in an ultracentrifuge to pellet the ECV. The pelleted ECV were resuspended in 100–200 μL of cold PBS

taking care not to disturb the pellet. Resuspend the pellet (ECV) in a limited volume (100–200 μL) of phosphate-buffered saline (PBS) or serum-free cell culture medium (*see* **Note 4**).

4. Transfer ECV-suspension into a sterile microfuge tube. Reserve a small aliquot of the sample to determine protein concentration.

3.3 Quantification of ECV Concentration

There are different procedures to quantify ECV; here we list the most popular ones.

1. Quantification of Total Protein Concentration. Mix the ECV suspension by pipetting, and withdraw 20 μL for protein assay. Mix sample with an equal volume (i.e., 20 μL) of 2× protein sample buffer lacking bromophenol blue. Boil the mixture for 5 min at 95 °C to lyse ECV. Protein concentration may be determined using a colorimetric method or a fluorimetric method; *see* **Note 5**.
2. Nanoparticle Tracking Analysis (NTA). This is a light scattering technique that is useful for rapid assessment of ECV size and number (*see* **Note 6**). This procedure has been criticized for the lack of standardization, but generally provides acceptable measurement of the size and number of ECV [16]. The potential causes of error related to size assessment include inaccurate measurement of temperature, incorrect assessment of viscosity and external vibration. The ECV concentration

assessment is dependent on factors such as camera, laser wavelength, depth of laser beam, cleanliness/wear of the metallized glass optical flat surface, duration of measurements, optical alignment, vibration, and operator proficiency. Take the precautions necessary for the specific NTA instrument available for your laboratory to get sustainable results from the different measurements.

3.4 Purity Assessment of ECV Preparation

To assess the purity of ECV preparations, you will require continuous access to a scanning or transmission electron microscope (*see* **Note 7**). To prepare ECV for scanning electron microscopy (SEM), use the following procedure. Immunoblotting can also achieve assessment of ECV purity (*see* **Note 8**).

1. Collect ECV from conditioned media, blood plasma, or serum using the multistep centrifugation procedure as described in Subheading 3.2.
2. Spread 50 μL of the ECV suspension on a cover slip.
3. Fix with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4.
4. Rinse 2× in 0.1 M sodium cacodylate buffer, pH 7.4, then post-fixed in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 1 h.
5. Dehydrate the sample through a graded ethanol series (50, 70, 70, 95, 95, 100%), 10–15 min for each step.
6. Expose the sample to critical point drying (*see* **Note 9**). Keep the samples immersed in 100% EtOH, then place the samples in wire baskets and transfer to a precooled (18 °C) chamber of the critical point dryer (Ladd Research Industries, Williston, VT). Seal the chambers and flush several times with liquid CO_2. Heat the CO_2 filled chamber to 39 °C and increase the pressure in the chamber to above 1100 psig to change CO_2 from liquid phase to gaseous phase. The gas should then to be vented slowly from the chamber until atmospheric pressure is reached; in this stage, the samples were dehydrated without surface tension damage.
7. Mount the coverslips onto SEM stubs with double-sided carbon tape and silver paste.
8. Expose the samples to sputter-coating with gold. The coating is achieved by placing the stubs in the chamber of a Polaron Model E5100 sputter coater (Polaron Equipment Ltd., Watford, Hertfordshire) and deposit approximately 20 nm of gold onto the stubs.
9. Inspect the specimens with the SEM. Verify the size of ECV under different magnifications to assess the homogeneity of your preparation. Take photographs for your reference (*see* **Note 10**).

3.5 Uptake of ECV by Recipient Cells

This protocol may be used to assess in vitro uptake of ECV by recipient cells. The functional outcome of ECV-treatment may be measured by western blotting, microscopy, cellular proliferation assays, or real-time PCR.

1. Grow recipient cells to 60% confluency in complete growth medium.
2. Wash cells three times with sterile, warmed PBS. Replace medium with serum-free medium for 24 h.
3. Add desired ECV concentration to fresh, pre-warmed serum-free medium (*see* **Notes 11** and **12**). Add directly to cell culture.
4. Perform a time course to determine optimal incubation time. In our experience, 24 h post-treatment works well, although this will depend on the functional assays being used.

4 Notes

1. The percentage of FBS is dependent on the cell type. The generally accepted percentage is 10%, but some cells grow better in 5% (see the data sheet for the cell line) or perform a simple experiment to find out the optimal concentration for your cells.
2. FBS is known to contain ECV, which may affect the yield and purity of ECV harvested from the conditioned medium. Using sterile technique, centrifuge FBS at 100,000×*g* (ultracentrifuge) for 16 h to remove all ECV. Use the depleted FBS to supplement your media.
3. Special precautions and training for the use of the ultracentrifuge are required, as any misuse of this equipment may result in personal injury or damage to this expensive machine.
4. For maximum ECV-retrieval, resuspend the ECV pellet repeatedly in a small volume (i.e., 3×50 μL).
5. The advantage of the latter assay includes a lower detection threshold and greater tolerance for high phospholipid concentrations.
6. A ratio of protein content determined from a protein assay and particle number determined by Nanoparticle Tracking Analysis may be used as a straightforward protocol for determining ECV purity [17]. A ratio of 1×10^{10} to 3×10^{10} particles per μg protein has been proposed. This ratio helps provide information on the amount of contaminating protein that is co-pelleted throughout the ECV isolation procedure.
7. Preparations for scanning electron microscopy are easier and less time consuming than the transmission electron microscopy preparation.

8. Purity of exosome preparations should be assessed using a panel of marker antibodies. Common marker proteins include CD9, CD63, CD81, Hsp70, Alix, TSG101, and Flotillin-1.
9. This procedure helps to preserve ECV surface morphology better than air dehydration. In this procedure, the water within the specimen will be replaced with an exchange fluid such as ethanol or acetone. The exchange fluid then will be replaced with liquid CO_2. The liquid CO_2 is then exposed to its critical point (31 °C and 74 bar) to be converted to the gaseous phase by decreasing the pressure at constant critical point temperature.
10. Although this procedure seems to be time consuming, in fact it is not, and if you have access to a reasonably good EM facility, this is one of their daily procedures. You need to use this procedure at least once when you begin collecting the ECV from the given specimen to familiarize yourself with the morphology and structure of ECV.
11. We recommend performing a quantitative dose–response curve to determine the optimal working concentration for your system.
12. It is important to use systematic negative controls to validate data obtained. For example, "mock ECVs" (e.g., culture medium that has not been conditioned by cells of interest) provide a baseline of background functional activity. ECVs from BS contain functional miRNAs and proteins that may impact measurements [18].

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References

1. Raposo G, Stoorvogel W (2013) Extracellular vesicles: exosomes, microvesicles, and friends. J Cell Biol 200:373–383
2. Lo Cicero A, Stahl PD, Raposo G (2015) Extracellular vesicles shuffling intercellular messages: for good or for bad. Curr Opin Cell Biol 35:69–77
3. Muralidharan-Chari V, Clancy JW, Sedgwick A, D'Souza-Schorey C (2010) Microvesicles: mediators of extracellular communication during cancer progression. J Cell Sci 123:1603–1611
4. Thery C, Zitvogel L, Amigorena S (2002) Exosomes: composition, biogenesis and function. Nat Rev Immunol 2:569–579
5. Valadi H, Ekstrom K, Bossios A, Sjostrand M, Lee JJ, Lotvall JO (2007) Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat Cell Biol 9:654–659
6. Montecalvo A, Larregina AT, Shufesky WJ, Stolz DB, Sullivan MLG, Karlsson JM et al (2012) Mechanism of transfer of functional microRNAs between mouse dendritic cells via exosomes. Blood 119:756–766
7. Thakur BK, Zhang H, Becker A, Matel I, Huang Y, Costa-Silva B et al (2014) Double-stranded DNA in exosomes: a novel biomarker in cancer detection. Cell Res 24:766–769

8. Montermini L, Meehan B, Garnier D, Lee WJ, Lee TH, Guha A, Al-Nedawi K, Rak J (2015) Inhibition of oncogenic epidermal growth factor receptor kinase triggers release of exosome-like extracellular vesicles and impacts their phosphoprotein and DNA content. J Biol Chem 290:24534–24546
9. Al-Nedawi K, Meehan B, Micallef J, Lhotak V, May L, Guha A, Rak J (2008) Intercellular transfer of the oncogenic receptor EGFRvIII by microvesicles derived from tumour cells. Nat Cell Biol 10:619–624
10. Al-Nedawi K, Meehan B, Rak J (2009) Microvesicles: messengers and mediators of tumor progression. Cell Cycle 8:2014–2018
11. Al-Nedawi K (2014) The yin-yang of microvesicles (exosomes) in cancer biology. Front Oncol 4:172
12. Lee TH, D'Asti E, Magnus N, Al-Nedawi K, Meehan B, Rak J (2011) Microvesicles as mediators of intercellular communication in cancer—the emerging science of cellular 'debris'. Semin Immunopathol 33:455–467
13. Costa-Silva B, Aiello NM, Ocean AJ, Singh S, Zhang H, Thakur BK et al (2015) Pancreatic cancer exosomes initiate pre-metastatic niche formation in the liver. Nat Cell Biol 17:816–826
14. Al-Nedawi K, Meehan B, Kerbel RS, Allison AC, Rak J (2009) Endothelial expression of autocrine VEGF upon the uptake of tumor-derived microvesicles containing oncogenic EGFR. Proc Natl Acad Sci U S A 106:3794–3799
15. Melo SA, Luecke LB, Kahlert C et al (2015) Glypican-1 identifies cancer exosomes and detects early pancreatic cancer. Nature 523(7559): 177-182. doi: 10.1038/nature14581
16. Soo CY, Song Y, Zheng Y et al (2012) Nanoparticle tracking analysis monitors microvesicle and exosome secretion from immune cells. Immunology 136(2):192–197. doi:10.1111/j.1365-2567.2012.03569.x
17. Webber J, Clayton A (2013) How pure are your vesicles?. J Extracell Vesicles 2:10. 3402/jev.v2i0.19861. eCollection 2013. doi:10.3402/jev.v2i0.19861
18. Shelke GV, Lasser C, Gho YS et al (2014) Importance of exosome depletion protocols to eliminate functional and RNA-containing extracellular vesicles from fetal bovine serum. J Extracell Vesicles 3:10.3402/jev. v3.24783.eCollection 2014. doi:10.3402/jev.v3.24783