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Article

Isolation and Characterization of Extracellular Vesicles derived from Human Umbilical Cord Mesenchymal Stem Cells

Running title: extracellular vesicles from human umbilical cord MSC

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Abstract

The safety and efficacy of mesenchymal stem cells/marrow stromal cells (MSC) have been widely studied. Since they are hypoimmunogenic, MSC can escape immune recognition, thus making them an attractive tool in clinical settings beyond autologous cell-based therapy. Paracrine factors including extracellular vesicles (EVs) released by MSC play a significant role in exerting therapeutic effects of MSC. Since their first discovery, MSC-EVs have been widely studied in an attempt to tackle the mechanisms of their therapeutic effects in various disease models. However, currently there are no standard methods to isolate EVs. Here, we describe a differential centrifugation-based protocol for isolation of EVs derived from human umbilical cord MSC (huc-MSC). In addition, the protocol describes methods for characterization of the EVs using transmission electron microscope, Western blot, and nanoparticle tracking analysis.

Keywords: extracellular vesicles, human umbilical cord, mesenchymal stem cells

1 Introduction

Mesenchymal stem cells have been the subject of interest in cellular therapy since Freidenstein first isolated the cells from the bone marrow of guinea pigs ***(1,2,3)***. MSC are not only able to self-renew but also to differentiate into various types of cells making them an attractive tool for regenerative medicine ***(4)***. Another intriguing feature of MSC is their ability to escape immune recognition and modulate immune cells in a wide range of diseases such as inflammatory bowel disease, traumatic brain injury, asthma, and chronic obstructive pulmonary disease ***(5,6,7,8)*** This immunomodulation is mediated by cell-to-cell contacts and via the release of paracrine factors such as cytokines, growth factors, and extracellular vesicles (EVs) ***(9)*** The paracrine effects of MSC were first observed in MSC cultured under different conditions. MSC cultured in normal growth medium, dexamethasone supplemented medium, and IL-1α supplemented medium were shown to express different cytokines profiles***(10)***. It was not until 2009 when Chen and colleague discovered EVs enriched with pre-microRNA in conditioned medium from embryonic stem cell-derived MSC ***(11)***.

EVs are heterogeneous particles involved in cellular communication that are released into the microenvironment by all cell types. Different terms have been used for EVs, including ectosomes, microparticles, prostasomes, oncosomes, membrane particles, migrasomes, and epididimosomes. To standardize the nomenclature of EVs, the International Society for Extracellular Vesicles (ISEV) suggests using the term “extracellular vesicle” unless the subcellular origin of the vesicle is demonstrated ***(12)***. The most widely studied types of EVs are exosomes and microvesicles. Exosomes are formed from inward budding of endosome that forms multivesicular bodies (MVBs) and are released when MVBs fuse with the cell

membrane ***(13)***. Exosomes are heterogeneous in size ranging from 40 to 100nm in diameter. Released exosomes can be taken up by other cells via endocytosis, fusion, or phagocytosis ***(14)***. Exosomes contain several specific markers such as tetraspanins (CD63, CD9, CD81, and CD82), flotillin, TSG101, and heat shock proteins (HSP60, HSP70, HSPA5, CCT2, and HSP90) ***(15)***. In contrast, microvesicles are formed by outward budding of the cell membrane and subsequently released to the extracellular space. The size of microvesicles ranges from 50 to 1000nm with integrins, selectins, and CD40 being the main protein markers of MVs ***(15)***. Recently, an increasing number of studies have been conducted to decipher the therapeutic effects of MSC-EVs in various diseases and conditions including, but not limited to, multiple sclerosis, diabetic nephropathy, myocardial infarction, and brain injury ***(16,17,18,19)***. These effects have been found to be mediated by various biomolecules including miRNA, protein, lipid, and RNA ***(20,21)***.

Ultracentrifugation remains the most utilised method to isolate EVs as the protocol is simple and can produce high yield of EVs ***(22)***. Characterization of EVs is usually conducted by a combination of morphological observation, protein marker analysis as well as by analysis of the size distribution ***(23)***. Our study has found that huc-MSC-derived EV reduced the inflammatory responses in animal model of chronic obstructive pulmonary disease (COPD) thus EVs could serve as a future cell-free therapy for the treatment of chronic lung diseases ***(24)***. This chapter describes the isolation of huc-MSC-EVs using differential centrifugation followed by their characterization using three independent methods. Morphology of huc-MSC-EVs was conducted using the transmission electron microscope (TEM); meanwhile, protein marker characterization and size distribution were conducted using western

blot and nanoparticle tracking analysis (NTA) respectively. The methods applied in this study can also be used to isolate and characterize EVs from other sources.

2 Materials

Reagents

1. Dulbecco's Modified Eagle Medium/F12 (DMEM/F12)
2. Fetal bovine serum (FBS)
3. L-glutamine
4. Antibiotic antimycotic
5. Basic fibroblast growth factor
6. 1.5M Tris/HCL
7. Ammonium Persulfate
8. Sodium Duodecyl Sulfate (SDS)
9. Acrylamide/Bis solution 37.5:1
10. N,N,N',N'-Tetramethylethylene-1,2-diamine (Temed)
11. Tween-20
12. Methanol
13. Rabbit monoclonal antibody β-actin (13E5)
14. Rabbit monoclonal antibody CD63 (EPR5702)
15. Goat polyclonal anti-rabbit IgG FITC
16. Bovine serum albumin
17. Phosphate-Buffered Saline

Equipment

- Biological safety cabinet Class II
- 15mL polypropylene conical centrifuge tube
- 50mL polypropylene conical centrifuge tube

- 75cm^2 flask
- NU-5510/E Air-Jacketed DHD Autoflow CO_2 Incubator
- 26.3mL polycarbonate aluminium bottle with cap assembly
- Allegra X-15R Ultracentrifuge
- Eppendorf centrifuge 5810R
- Type 50.2Ti fixed-angle rotor, Optima L-100K Ultracentrifuge
- Carbon-coated copper grids
- Uranyl Acetate
- Energy filtered transmission electron microscopy
- Nanosight NS300
- Polyvinylidine difluoride (PVDF) membrane
- Mini-PROTEAN Short Plate
- Mini Trans-Blot Cell
- Li-Cor Odyssey Fc Dual Mode Imaging System

2.1 EV-free FBS medium

Supplement Dulbecco's Modified Eagle Medium/F12 (DMEM/F12) with 20% fetal bovine serum (FBS) and centrifuge using Type 50.2Ti fixed-angle rotor, Optima L-100K Ultracentrifuge for 18 hours at 100,000 x g (see Note 1). Collect the supernatant from the ultracentrifuge tube and discard the pellet. Prepare 10% FBS-EV free DMEM/F12 by diluting with DMEM/F12 and supplement with 1% antibiotic antimycotic and 1% L-glutamine in EV-free FBS medium.

2.2 Cell Culture Medium

Supplement DMEM/F12 with 10% FBS, 1% antibiotic antimycotic, and 1% L-glutamine.

2.3 SDS polyacrylamide gel

2.3.1 12% separating gel 10mL

Mix 2.5mL of 1.5M Tris/HCL pH 8.8 with 100µL of 10% SDS, 100µL of 10% ammonium persulfate (see Note 2), 3mL of 40% acrylamide/Bis solution 37.5:1, 4.3mL of ddH_2O, and 2.5µL of N,N,N',N'-Tetramethylethylene-1,2-diamine (Temed) (see Note 3).

2.3.2 3% stacking gel 5mL

Mix 1.25mL of 0.5M Tris/HCL pH 6.8, 50µL of 10% SDS, 50µL of 10% Ammonium Persulfate, 375µL of 40% acrylamide/Bis solution 37.5:1, 3.275mL of ddH_2O, and 2.5µL of Temed (see Note 3).

2.4 Running buffer (1x)

Add 100mL of 10x Running Buffer and 10mL of 10% SDS in 890mL ddH_2O. Prepare 1L of 10X Running Buffer 1L by mixing 30.3g of Tris/HCL (trizma Base) Mr 121.14, pH 8.3, and 188g of Glycine in ddH_2O.

2.5 Transfer buffer (1x)

Add 50mL of 20x Transfer buffer, 200mL of methanol, and 750mL of ddH_2O. Prepare 1L of 20x Transfer Buffer by mixing 116.3g of Tris/HCL (trizma Base) Mr 121.14, 58.6g of Glycine, 7.4g of SDS in ddH_2O

2.6 Antibodies

Mix rabbit monoclonal antibody β-actin in 5mL of 1x PBS with 2% BSA (see note 4).

Mix rabbit monoclonal antibody CD63 in 5mL of 1x PBS with 2% BSA

Mix goat polyclonal anti-rabbit IgG in 5mL of 1x PBS with 2% BSA

2.7 Blocking Buffer

Mix 10mL of 1x PBS and 0.2g of BSA to prepare 2% BSA. Blocking buffer should be prepared fresh for every use.

2.8 2% Uranyl Acetate

Add 1g of uranyl acetate in 40mL of distilled water in a 50mL amber bottle with cap. Stir the solution slowly until fully dissolve. Top up the solution with distilled water to 50mL. Keep in 4^{o}C.

3 Methods

3.1 Culture of huc-MSC

1. Cultivate 4000 cells/cm^2 of huc-MSC in 10mL cell culture medium with 20ng/mL basic fibroblast growth factor (bFGF) added. Incubate the flask in a humidified incubator at 37^oC supplied with 5% carbon dioxide.
2. Maintain the cells in 75cm^2 flask. After 48 hours, change the medium to EV-free FBS medium to collect huc-MSC-EVs. Add 20mL of EV-free FBS medium into the flask and incubate in the humidified incubator at 37^oC supplied with 5% carbon dioxide.

3.2 Isolation of huc-MSC-EVs

1. Collect the conditioned medium from the 75cm^2 flask after 72 hours and transfer to a 50mL polypropylene conical centrifuge tube.
2. Centrifuge the conditioned medium at 300xg for 10 minutes to remove dead cells.
3. Collect the conditioned medium into a new 50mL polypropylene conical centrifuge tube and centrifuge at 10,000xg for 30 minutes in an ultracentrifuge to remove apoptotic bodies.
4. Collect the supernatant into a polycarbonate aluminium bottle and centrifuge using ultracentrifuge at 100,000xg for 2 hours using Type 50.2Ti fixed-angle rotor to concentrate huc-MSC-EVs.
5. Discard the conditioned medium. Add 23mL of 1x PBS in the polycarbonate aluminium bottle containing huc-MSC-EVs and centrifuge again using an ultracentrifuge at 100,000xg for 2 hours using Type 50.2Ti fixed-angle rotor to wash the EVs.

6. Discard the supernatant and add 150uL of 1x PBS in the tube. Gently resuspend the huc-MSC-EVs and collect the huc-MSC-EVs in 0.5mL microcentrifuge tube.

3.3 Transmission Electron Microscope Analysis

1. Load the freshly isolated huc-MSC-EVs in 1x PBS (see Note 5) onto the carbon-coated copper grids and incubate for 10 minutes.
2. Blot the grid with filter paper to remove the excess liquid. Stain the EVs with 2% uranyl acetate for 1 minute.
3. Remove the excessive uranyl acetate and let the grid dry for 15 minutes before viewing using an appropriate electron microscope (see Figure 1 for example image ***(24)***).

3.4 Nanoparticle Tracking Analysis

1. Dilute the huc-MSC-EVs with 1x PBS to the concentration of 20 to 60 particles per field (see Note 6).
2. Load the sample using a 1mL syringe into the nanoparticle tracking analysis Be careful not to formed bubble as bubbles will interfere with the reading (see Figure 2 and Table 1 for example readouts).
3. Record five readings for each huc-MSC-EV sample.

3.5 Western Blot

1. The gel should be prepared a day before running the western blot.
2. Add 7.5mL to the casting system and gently layer 250uL of water-saturated isobutanol (see Note 7).

3. When the gel has set, drain the water-saturated isobutanol and gently dry with filter paper.
4. Layer with 3% Stacking gel to the top and add in the comb. Be careful not to form bubbles.
5. Keep at 4^{o}C overnight.

3.6 Gel electrophoresis

1. Mix sample with 8uL of loading dye and denature the sample at 100^{o}C for 3 minutes.
2. Cool the sample and centrifuge at 16,000 rpm for 1 minute.
3. Load the sample in the gel and fill the tank with 1x running buffer and run at 20 mA for 3 hours.

3.7 Transfer Protocol

1. Cut the PVDF membrane to the size of 9 x 7.5 cm.
2. Soak it in methanol for 15 sec and ddH_2O for 2 min.
3. Equilibrate in 1x transfer buffer for 10 minutes.
4. All sponges, filter paper must be soaked in 1x transfer buffer.
5. Layer the sandwich on the cassette and place the cassette in the mini-trans blot cell.
6. Fill the cooling unit with dry-ice (see Note 8), fill with transfer buffer, and a stirring bar and place on stirring unit.
7. Run at constant current, 1 amp per cell, for 1 hour.
8. Layer the PVDF membrane on the tissue and dry it at 37^{o}C for 10 minutes to make sure the protein sticks to the membrane properly.

9. Rehydrate the membrane in methanol for 15 seconds, and ddH_2O for 5 seconds. Transfer the membrane to 1x PBS for 1 min.
10. Add blocking buffer and incubate for 1 hour at room temperature on a rocker.
11. Add primary antibody and incubate overnight at 4^oC on a rocker.
12. Wash the membrane 6 times with 1x PBST (see Note 9) 5 min each washing.
13. Incubate with secondary antibody for 1 hour at room temperature on a rocker.
14. Wash the membrane 6 times with 1x PBST 5 min each washing.
15. Ready to view with suitable imaging system (see Figure 3 for example blot ***(24)***).

4 Notes

1. Centrifugation of medium containing EVs for 18 hours will remove 95% of the EVs from the medium.
2. 10% ammonium persulfate should be prepared fresh in dH_2O.
3. Add Temed prior to loading the gel in the gel cast to avoid solidification of the mixture before gel loading.
4. Dilute the antibody as per manufacturer's suggestion.
5. Use freshly prepared 1x PBS to avoid precipitation that will interfere with the viewing.
6. The dilution can be done gradually. When too many particles are seen in the field, stop the machine, and dilute the sample until the desired number of particles in the field are achieved.
7. Mix equal parts of isobutanol and dh_2O and keep in room temperature for further use.
8. Dry-ice is used to cool off the mini trans-blot cell as running at 1 amp will produce heat.
9. Add 1mL of tween-20 in 999mL of 1x PBS. Mix well and keep in 4^oC.

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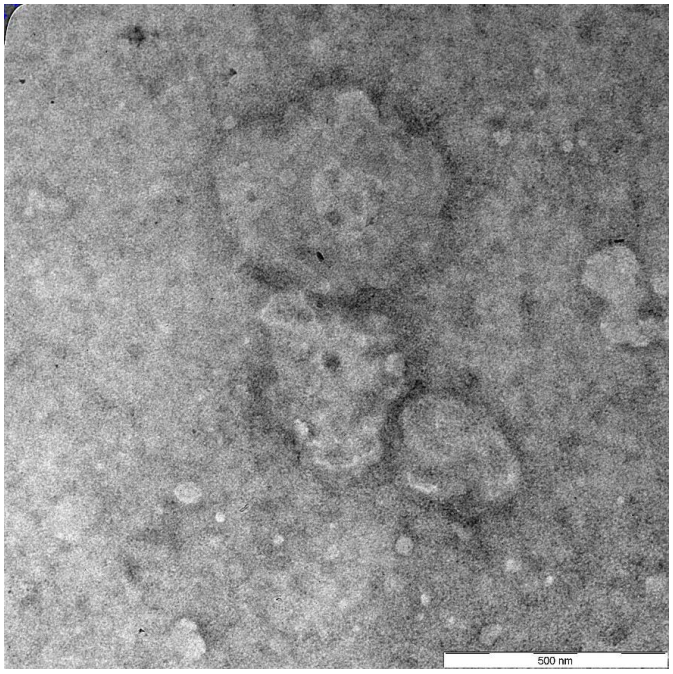

Figure 1. Morphological observation of huc-MSC-EVs using TEM.

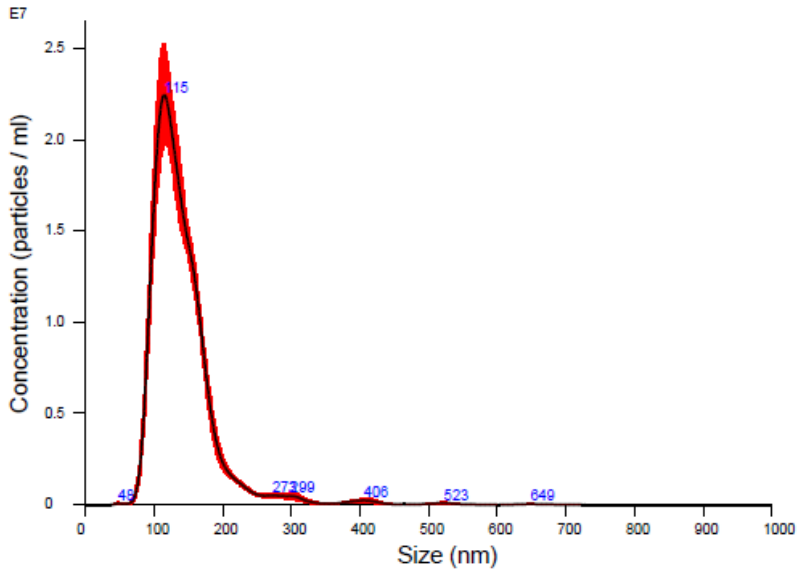

Figure 2. Size distribution analysis of hUCMSC-EV. Particle distribution by Nanosight NS300 reported an average MSC-EV diameter of 153nm. The graph represents 3 independent experiments.

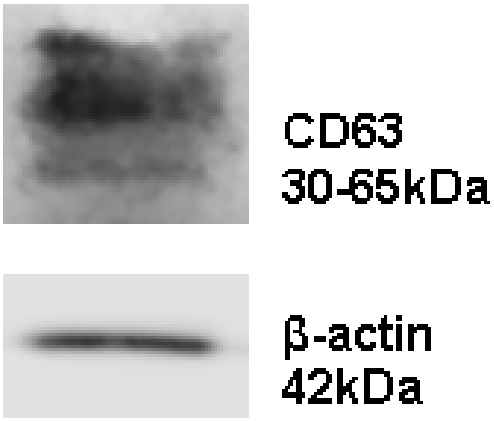

Figure 3. Expression of protein marker using western blot. β-actin and CD63 were assessed on UCMSC-EV.

Table 1: Analysis of hUCMSC-EV size distribution

Sample	Mean (nm)	Mode (nm)	SD (nm)	Range (nm)
1	141.2	115.0	51.4	36 - 737
2	156.5	116.9	68.4	64-795
3	163.0	123.1	68.3	25-740

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