Characterization of human cardiac mesenchymal stromal cells and their extracellular vesicles comparing with human bone marrow derived mesenchymal stem cells

Running title; human cardiac mesenchymal stromal cells and their EVs

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SUPPLEMENTARY INFORMATION

MATERIALS AND METHODS

Human cells

Human cardiac mesenchymal stromal cells (h-CMSCs) were explanted from cadaveric tissue and expanded which were as described previously (1). Briefly, fibrin-supported 3D myocardial organ cultures were performed under dynamic conditions at 15 rpm after removing epicardium and endocardium. The myocardium was minced into 2-3 mm³ fragments and washed with phosphate-buffered saline (PBS). After 7 d of the culture, outgrown cells were collected and suspended in growth culture media for a conventional monolayer culture condition. When cells reached 80% confluence, the cells were detached and subcultured. Human bone marrow derived mesenchymal stem cells (h-BM-MSCs) and h-CMSCs were provided by Young Il Yang M.D (Paik Institute for Clinical Research, Inje University College of Medicine, Busan, Republic of Korea). Human umbilical cord blood cell (HUVEC)s were purchased from Lonza (MD, USA).

For h-CMSC culture, 1:1 mixture of Dulbecco’s Modified Eagle’s Medium: Nutrient Mixture F12 (DMEN/F12) (Gibco, USA) with epidermal growth factor (EGF) 10 ng/mL, insulin-growth factor (IGF) 10 ng/mL, basic fibroblast growth factor (bFGF) 2 ng/mL and gentamycin 10 µg/mL were used. One to one mixture of DMEN/F12 with 10% fetal bovine serum (FBS) (Thermo Scientific, USA) and gentamycin 10 µg/mL were used for MSC culture. For HUVEC culture, EBM-2 basal medium supplemented with the EGM-2 SingleQuots supplement kit (Cat.No.: CC-3162, Lonza, MD, USA) and 2% FBS were used on the gelatin coated dishes. At passage 12 were used for the experiments.
Hypoxic treatment was performed with human CMSCs for hypoxic CMSC EVs by using Hypoxia Incubator Chamber (Cat.No.: 27310, STEMCELL™, Canada) under the condition of 2% O₂, 5% CO₂ and 93% N₂.

**Reverse transcriptase polymerase chain reaction (RT-PCR) assays**

For total RNA extraction, the cells were lysed in TRIzol (Invitrogen, Thermo Fisher Scientific, NH, USA). After adding chloroform and centrifuging and add isopropanol to the supernatant containing the RNA. Centrifuging after standing at room temperature for 5 min, add 75% ethanol to the pellet. Add diethyl pyrocarbonated treated water to the pellet after another centrifugation. The purity of isolated RNA was determined by OD260/280 using a DeNovix DS-11 spectrophotometer (DeNovix, DE, USA).

1 μg of extracted RNA of each group was used as a template for first-strand cDNA synthesis by reverse transcription (RT) with RNase inhibitor and dNTP mix (Thermo Fisher Scientific, NH, USA). PCR was performed for 40 cycles with gene-specific primer sequences (Table) and AccuPower PCR premix (Bioneer, Daejeon, Republic of Korea). The following PCR conditions were used: 50°C for 2 min and 95°C for 10 min, followed by 40 cycles for 30 s at 95°C, 45 s at 60°C and 72°C for 30 s. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control.

**Extracellular vesicles isolation and characterization**

Isolation of EVs

EVs from 72 h the medium conditioned either with BM-MSCs, normoxia or hypoxia-treated CMSCs were isolated using ExoLutE® exosome isolation kit (Rosetta Exosome Inc, Seoul, Republic of Korea). Briefly, the medium harvested from the culture was differentially centrifuged at 500 x g for 10 min and 2,000 x g for 15 min, respectively. To enrich EVs from
the pre-cleared conditioned medium as described previously (2), the medium was treated with polyethylene glycol 6000 (Sigma-Aldrich, MO, USA) final at 8.3% then incubated at 4°C for 16 h. After centrifugation at 13,000 x g for 10 min, the pellet was resuspended in 8 mL of RPMI1640 medium (Gibco, NH, USA) to purify EVs further. According to the manufacturer’s instruction of ExoLutE® exosome isolation kit, the highly pure EVs were finally isolated by spin-based size-exclusion chromatography which was pre-equilibrated with HEPES-buffered saline (22 mM HEPES, 150 mM NaCl, pH7.4). Protein quantity in the EV preparation was measured by QuantiProTM BCA assay kit (Sigma-Aldrich, MO, USA) and aliquots of the purified EVs were frozen by liquid nitrogen then stored at -80°C until necessary.

Transmission electron microscopy
Purified EVs were subjected on transmission electron microscopy to determine their shapes. 5 µl of EVs preparation at 1x10⁹ particles ul⁻¹ was adsorbed onto glow-discharged carbon-coated copper grids (Electron Microscopy Sciences, PA, USA) for 5 min. Following the removal of excess liquid, the grid was washed 10 times with PBS and subsequently stained with 2% uranyl acetate (Ted Pella, Redding, CA, USA). The grid was examined in JEM 1011 microscope (JEOL, Tokyo, Japan) and images were recorded with an ES1000W Erlangshen CCD Camera (Gatan Inc. Pleasanton, CA, USA).

Measurement of EV size distribution
The size distributions of purified EVs were measured with Zetasizer Nano ZS (Malvern Instrument Ltd., Malvern, UK). The size distribution was determined by the light scattering against infra-red light (wavelength = 633 nm) with Dynamic V6 software. Results are mean values from five measurements.
Nanoparticle tracking analysis

The particle concentrations of purified EVs were determined using a Nanosight LM10-HS system (Nanosight Ltd., Amesbury, UK). Appropriately diluted purified EVs were injected into the chamber and visualized using a 405 nm laser. The recorded images with a camera level at 12 were analyzed using the nanoparticle tracking analysis (NTA) software (version 2.3) with a threshold at 5.

Size-exclusion chromatographic analysis

The chromatograms of purified EVs were analyzed by a column packed with Sephacryl S500 (GE Healthcare Life Sciences, USA) which was connected on a high-pressure liquid chromatography system (Thermo Scientific, UltiMate™ 3000, USA). HEPES-buffered saline (20 mM HEPES, 300 mM NaCl, pH7.4) was used as a mobile phase with a flow rate at 1.0 ml/min. 280 nm wavelength was recorded to analyze the purity of isolated EVs and 1 µg of purified EVs was used for each test.

Western blotting

Whole cell lysates (20 µg of total protein) and corresponding purified EVs (2 µg of total protein) were loaded onto SDS-PAGE gels (4-20%, Bio-Rad, CA, USA), and then transferred to polyvinylidene difluoride membranes (Millipore, MA, USA). The membrane was blocked by 3% skim milk and incubated at 4°C for overnight either with primary antibodies (mouse anti-human CD9; BD Biosciences, Cat. No.: 555370, 1:1000, mouse anti-human CD63; BD Biosciences, Cat. No.: 556019, 1:1000, and mouse anti-human CD81; Santa Cruz, Cat. No.: sc-166029, 1:500). After incubation, the membrane was followed by the incubation with corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies. The
immunoreactive bands were visualized using enhanced chemiluminescence substrate (Thermo Scientific, NH, USA).

**Flow cytometry**

Cells were incubated with an anti-CD16/32 monoclonal Ab (BioLegend, CA, USA) at 4°C for 30 min (to block the Fc receptors). Cells were stained with specific antibodies against the CD34, CD45, CD90, CD105 and CD117 (Cat. No.: 343514, 368505, 328107, 323217 and 313203, respectively, BioLegend. CA, USA) surface markers in FACS buffer (PBS containing 2% FBS) at 4°C for 30 min, and washed with FACS buffer. Stained cells were acquired using a BD LSRFortessa flow cytometer and analyzed with the FlowJo software (Tree Star, CA, USA).

**Tube formation assay**

HUVECs were plated on Matrigel coated (75 µl per well) 96-well plates at a density of 1.25 x 10^4 cells per well. Three types of EVs from BM-MSC, normoxia and hypoxia-treated CMSCs were added to the well with EGM media at a dose of about 0.4 µg/well (2 µg/ml; 2.1 x 10^9 particles/µg BM-MSC, 1.9 x 10^9 particles/µg normoxic CMSC, 1.3 x 10^9 particles/µg hypoxic CMSC). HUVECs with EGM media only (negative control: NC) and HUVECs with EGM plus vascular endothelial growth factor (VEGF, 20 ng/ml) were used for negative and positive control. All wells were triplicated. Images (x 5) were obtained under light microscopy 4, 6 and 8 h after cells with EVs plating. Quantification of total tube length was performed using Image J (NIH, MD, USA).

**Statistical analysis**

Statistical analyses were performed using GraphPad Prism (La Jolla, CA, USA) with the threshold for significance set at level P < 0.05. Values are expressed as mean ± standard
error. To compare tube length of each groups, we used the one way ANOVA followed by the Tukey’s post hoc analysis for all pairwise comparisons.
| Gene     | Forward primer (5’ to 3’)                        | Reverse primer (3’ to 5’)                      |
|----------|------------------------------------------------|-----------------------------------------------|
| GATA4    | GACGGGTCACTATCTGTGCAAC                           | AGACATCGCACTGACTGAGAAC                       |
| NKX 2-5  | CGCCCTTCTCAGTCAAAGAC                            | AGATCTTGACCTGCCTTGAC                        |
| MYH6     | GTCATTGCTGAAACCGAGAATG                          | GCAAAGTACTGGATGACACGCT                      |
| NANOG    | AGTCCCAAAGGGCAAACACCCACTTC                      | TGCTGGAGGCTGAGGTATTCTCTGTCTC                |
| SOX2     | ATGCACCGCTACGACGTGA                             | CTTTTGCACCCCTCCCATTT                       |
| GAPDH    | AAGTGGATATTGTTGCCATC                            | ACTGTGGTCATGAGTCTCTC                        |
Supplementary Figures

Supplementary figure 1. Yields of extracellular vesicles per million cells; yields of extracellular vesicles per million cells were highly variable according to the cell types.

Supplementary figure 2. Tube formation according the concentration of EVs

Normoxic CMSCs EVs showed increasing tube formation according to the concentration of EVs in low dose but decreased at higher dose 1.0 µg/well (A) and similar findings were observed in hypoxic CMSCs EVs (B). NC, negative control; ** P < 0.01 vs. NC
References

1. Kim JT, Chung HJ, Seo JY et al (2015) A fibrin-supported myocardial organ culture for isolation of cardiac stem cells via the recapitulation of cardiac homeostasis. Biomaterials 48, 66-83

2. Lee C, Mitsialis SA, Aslam M et al (2012) Exosomes mediate the cytoprotective action of mesenchymal stromal cells on hypoxia-induced pulmonary hypertension. Circulation 126, 2601-2611