Regulation of alternative macrophage activation by MSCs derived hypoxic conditioned medium, via the TGF-β1/Smad3 pathway

Ran Kim[1], Byeong-Wook Song[2], Minji Kim[1], Won Jung Kim[1], Hee Won Lee[1], Min Young Lee[3], Jongmin Kim[4] &Woochul Chang[1,]*

[1]Department of Biology Education, College of Education, Pusan National University, Busan 46241,
[2]Department of Medical Science, College of Medicine, Catholic Kwandong University, Gangneung 25601,
[3]Department of Molecular Physiology, College of Pharmacy, Kyungpook National University, Daegu 41566,
[4]Department of Life Systems, Sookmyung Women's University, Seoul 04310, Korea

Supplemnetary Meterials and Methods

Animals

C57Bl/6 male mice (5-8 weeks old) were used in the experiments (KOATECH, Gyeonggi-do, Korea). The mice were housed under constant conditions at temperature 25 ± 1°C, humidity 50 ± 10%, and 12 h light/dark cycle, with free access to food and water. All experiments were performed according to the guidelines issued by the Korean National Institute of Health and the Korean Academy of Medical Sciences for the care and use of laboratory animals.

Isolation and culture of bone marrow derived cells

For isolation of BM derived MSCs, BM were flushed with complete MesenCult™ Medium (Stem Cell, Vancouver, Canada) and centrifuged. The resultant pellet was resuspended and plated in MesenCult Medium with Mesenpure™ (Stem Cell). After 3 days, the plates with attached cells were washed and replenished with fresh medium. Isolation of BMDMs was achieved by disassociating the attached cells using DPBS with 0.5M EDTA (Sigma-Aldrich, MO, USA), followed by centrifugation. The resultant pellet was resuspended and incubated in RBC lysis buffer (Biosesang, Gyeonggi-do, Korea), and subsequently neutralized with DPBS. BMDMs were cultured in Dulbecco's Low Glucose Modified Eagles Medium (Low

DMEM; Hyclone, UT, USA) supplemented with 10% FBS (Hyclone) and recombinant murine M-CSF (Peprotech, Seoul, Korea).

Reverse transcription polymerase chain reaction

Total RNA was extracted using the Hybrid-R RNA purification kit (GeneAll, Seoul, Korea). RNA was converted to cDNA using Solg™ Taq DNA Polymerase (SolGent, Daejeon, Korea), according to the manufacturer's protocol. The relative mRNA expression was calculated using the ImageJ software.

Western blotting

Cells were collected and lysed for isolation of proteins. Total proteins were quantified by subjecting to SDS-PAGE, and subsequently transferred to PVDF membranes (Millipore, MA, USA). The membranes were incubated with the following primary antibodies: β-actin, HIF-1α, STAT6 and p-STAT6 (1:1000; Cell Signaling Technology, MA, USA), TGF-β1, Smad3 and p-Smad3 (1:1000; Abcam, Cambridge, UK).

Preparation of hypoxic conditioned medium derived from MSCs

MSCs were plated in 60 mm plates at a density of 9×10^5 cells per well. The plates were washed and replaced with fresh medium. MSCs were then cultured for 12 h, in serum free (SF)-low DMEM under 1% O_2, 5% CO_2 blended with N_2. Hypoxic condition was provided by an anaerobic system (Forma scientific, Ontario, Canada).

Cell Proliferation Assay

BMDMs were seeded in 60 mm plates at a density of 2×10^6 cells per well. The plates were divided into 5 groups: group Ⅰ, BMDMs treated with 10% FBS/high DMEM; group Ⅱ, BMDMs treated with SF-high DMEM; group Ⅲ, BMDMs treated with hypo-CM derived from MSCs; group Ⅳ, BMDMs treated with 10% FBS/high DMEM and 30ng/ml of IL-4; group Ⅴ, BMDMs treated with 10% FBS/high DMEM, 20ng/ml of IFN-ɣ and 100ng/ml of LPS.

Flow cytometry analysis

To determine macrophage polarization, BMDMs were resuspended in FACS buffer

and stained with APC-conjugated anti-mouse F4/80 (BioLegend, CA, USA), and either PE-conjugated anti-mouse CD86 (BioLegend) or FITC-conjugated anti-mouse CD71 (BioLegend). Cells were then identified using FACS CANTO Ⅱ (BD Biosciences, CA, USA), and data was analyzed by the FlowJo program.

Tube formation assay

BMDMs (1.5×10^5 cells per well) were seeded into inserts of 24-well plate and incubated for 24 h. HUVECs were seeded at a density of 3×10^4 cells per well in Matrigel coated 24-well plates. BMDM inserts were then introduced in the HUVEC wells. The plates were divided into 4 groups: group Ⅰ, HUVECs treated with SF-high DMEM; group Ⅱ, HUVECs treated with 10% FBS/high DMEM and 20ng/ml of recombinant human $VEGF_{165}$ (Peprotech, Seoul, Korea); group Ⅲ, HUVECs co-cultured with BMDMs treated with normoxic-CM derived from MSCs; group Ⅳ, HUVECs co-cultured with BMDMs treated with hypo-CM derived from MSCs.

Myocardial infarction surgery

MI surgery was conducted as previously described (1). Briefly, under positive-pressure ventilation, the ribs of mice were cut to open the chest, and heart was exteriorized through the intercostal space. The left descending coronary artery was permanently ligated with 8-0 prolene suture. The ribs and skin were closed using 6-0 prolene suture. Occurrence of MI was assured by blanching of the myocardium and dyskinesis of the ischemic region.

TUNEL assay

Staining was performed as previously described (1). After de-paraffinization, sections of the heart tissue were treated with 20 μg/ml of Proteinase K (Sigma-Aldrich). Specimens were then washed in water, and quenched in 3% hydrogen peroxide. The samples were treated with terminal deoxynucleotidyl transferase enzyme in a humidified chamber at 37°C. Counter-staining was achieved using 0.5% methyl-green solution, followed by sequential dehydration using 100% N-butanol, ethanol and xylene.

Masson's trichrome staining

Collagen was demonstrated on formalin-fixed specimens embedded in paraffin and stained with Masson's trichrome reagent (Abcam). Briefly, the specimens were fixed in Bouin's liquor, followed by overnight incubation, and washed in running water until the yellow color disappeared. Sections were subsequently stained with Mayer's hematoxylin, placed in 0.5% hydrochloric acid in 70% alcohol, stained with acid ponceau, and dissolved in 1% phosphomolybdic acid solution. This was followed by brilliant green staining dissolved in 1% glacial acetic acid, and dehydrated in 95% ethyl alcohol. The tissues stained blue for collagen fibers, and red for cytoplasm, muscle fibers and red blood cells.

Immunohistochemistry

For assessing microvessel density, sections were incubated in PBS supplemented with 2% FBS and stained with antibody against CD31 (1:40, Dako, CA, USA), incubated overnight. Anti-mouse secondary antibody was used with HRP conjugation kit (Abcam). The specimens were stained with H&E. The number of microvessels was microscopically counted in 5 randomly chosen fields per a section (×200).

Statistical Analysis

All data were expressed as means ± SEM. Comparisons between more than two groups were analyzed by one-way ANOVA using Bonferroni's correction. A p-value < 0.05 is considered significant.

Reference

1. Lee SY, Lee S, Choi E et al (2016) Small molecule-mediated up-regulation of microRNA targeting a key cell death modulator BNIP3 improves cardiac function following ischemic injury. Sci Rep 6, 23472