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Integrin alpha5beta1 suppresses rBMSCs anoikis and promotes nitric oxide production

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ABSTRACT

Background: Cell based therapy has been heralded as a novel, promising therapeutic strategy for cardiovascular diseases including pulmonary arterial hypertension (PAH). However, the low survival rate after transplantation due to cell death via anoikis is a major obstacle in stem cell therapy. Cells adhesion via Integrin alpha5beta1 (ITGA5B1) has a tendency to exert higher maximum forces. The present study aimed to evaluate the potential protective effect of ITGA5B1 on rat bone marrow mesenchymal stem cells (rBMSCs) from anoikis.

Methods: Mononuclear cells were isolated by density gradient centrifugation with Ficoll, and rBMSCs cell surface markers were evaluated by flow cytometry. Osteogenic and adipocyte differentiation was determined by Alizarin Red S and Oil Red O staining respectively. The expression of Integrin A5 (ITGA5), Integrin B1 (ITGB1), eNOS and actived-caspase-3 mRNA or protein was confirmed by qPCR and western-blot. Cell adhesion, cell viability, anoikis and the migration of rBMSCs were also evaluated. Nitric oxide (NO) production was detected by the greiss assay.

Results: Co-infected with Integrin A5 and B1 lentivirus to rBMSCs increased ITGA5 and ITGB1 mRNA and protein expression. ITGA5B1 enhanced the cell adhesion, cell viability, cell migration and NO production but reduced the cell anoikis in rBMSCs/ITGA5B1 groups.

Conclusion: Transduction of rat rBMSCs with ITGA5B1 lentivirus could prevent cell anoikis and increase NO production.

1. Introduction

Pulmonary arterial hypertension (PAH) remains a rapidly progressive and eventually lethal disease [1]. Stem cells, characterized by their self-renewal and multilineage differentiation potential, highlight their advantages and make them an attractive candidate for clinical applications include PAH. However, cell survival rate is typically less than 1% after transplantation [2]. The low survival and engraftment rate after transplantation are due to cell death via anoikis driven by the loss of cell adhesion, which limits the successful application of stem cell therapy [3–6]. Normal cells are sensitive to anoikis [7], and our previous studies mainly focused on the treatment effect of bone marrow derived mesenchymal stem cells (BMSCs) on PAH rat [8,9], therefore, preventing cell anoikis to enhance the adhesion ability of stem cells at the injured site is important for promoting its survival and engraftment.

Anoikis is a cell detachment-induced apoptosis, which occurring upon cell detachment from the extracellular matrix, thus disrupting integrins ligation [10]. Integrins, which involved in cell-extracellular matrix interaction and connexins, regulate cellular adhesion and transmit signals important for cell survival, proliferation, gap junction and motility [11], and can resolve the poor survival of donor cells [12].

It is reported that integrins in pulmonary artery smooth muscle cells are differentially regulated in pulmonary hypertension, the level of alpha 5 and beta 1 integrins were reduced in PAH [13]. However, cell adhesion via integrin alpha5beta1 (ITGA5B1) has a tendency to exert higher maximum forces [14]. Furthermore, integrin induces endothelium dependent vasodilation by endothelial nitric oxide (NO) pathway [15]. More importantly, NO not only is one of therapeutics to treat PAH [16,17], it also can enhance the anoikis resistance and migration [18]. Therefore, the present study investigated the cell

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characteristics of the rat bone marrow derived mesenchymal stem cells (rBMSCs) transduced with ITGA5B1, in particular cell adhesion, anoikis, cell migration and NO production.

2. Materials and methods

2.1. Reagents

The lentiviral packaging plasmids psPax2, VSV-G and pRSV-Rev were given as a gift by Dr Padraig Strappe (Central Queensland University, Australian). The pLVX-mCMV-mCherry lentiviral vector backbone and the Integrin A5 (ITGA5), Integrin B1 (ITGB1) gene overexpressed lentiviral vector pLVX-mCMV- ITGA5-mCherry (LV-ITGA5) and pLVX-mCMV-ITGB1-mCherry (LV-ITGB1) vectors were purchased from Biowit Technologies Co., Ltd. (Shenzhen, China). CytoSelect™ 24-Well Anoikis Assay was purchased from Cell BioLabs.

2.2. rBMSCs isolation, culture and characterization

BMSCs were extracted from the bone marrow of bilateral femora and tibias of adult male rats (certificate number SCXK (Shandong) 20140007) ranging in weight from 120 to 160 g. Flushed cells were subjected to Ficoll (1.077 g/ml) density gradient separation, and the mononuclear fraction was harvested. This fraction was plated on T-25 plastic flasks with complete DMEM/F-12 (Gibco) culture medium and incubated in a humidified atmosphere containing 5% CO_2 at 37 °C. Nonadherent cells were removed after 3 days, and media changes were made at 3-day intervals. When primary cultures reached 70%–80% confluence, the cells were passaged by trypsinization and cultured in the above compound. All experimental procedures were approved by the local Research Ethics Committee (Liaocheng People's Hospital, Shandong, China) and conducted in strict conformity with local institutional guidelines and international standards for the manipulation and care of laboratory animals.

Cell specific surface markers were examined by flow cytometry: anti-CD34-PE (Santa Cruz, Dallas, TX, USA), anti-CD29-BV421™, anti-CD31-PE and anti-CD90-FITC (all from BD Biosciences PharMingen, San Diego, CA, USA) antibodies. The data analysis was conducted by the BD FACSDiva software. Each experiment was repeated for three times.

At the second passage, cells were subjected to osteogenic and adipocyte differentiation assays. For osteogenic differentiation, rBMSCs were incubated in DMEM and 10% FBS supplemented with dexamethasone (100 nM), beta-Glycerol phosphate (10 mM) and ascorbic acid 2-phopshate (200 μM, all from Sigma–Aldrich, St. Louis, MO, USA). The media were changed every 3 days. Osteocyte formation was detected by staining calcium deposits in the extracellular matrix with 2% Alizarin Red S (Sigma-Aldrich) after 14 days of osteogenic differentiation. Extracellular calcium deposits were observed under the microscope and stained bright orange-red. For adipocyte differentiation assay, rBMSCs (2×10^5/ml) were incubated with adipogenic induction media (complete DMEM) supplemented with dexamethasone (1 μM), 3-isobutyl-1-methylxanthine (IBMX, 0.5 mM), insulin (10 μg/ml), rosiglitazone (0.5 μM), and indomethacin (100 μM, all from Sigma–Aldrich, St. Louis, MO, USA) for 3 days and one day incubated with insulin (20 μg/ml) for 14 days. Cells differentiation to adipocytes were confirmed by Oil Red O (Sigma-Aldrich, St. Louis, MO, USA) staining and visualized by light microscopy.

2.3. Lentiviral vector production and transduction

HEK 293T human embryonic kidney cells (ATCC) and rBMSCs were cultured in Dulbecco's Modified Eagle's Medium (DMEM) and DMEM-F12 respectively, supplemented with 10% FBS (Hyclone), 100 U/ml penicillin, 100 μg/ml streptomycin, and were cultured in a humidified atmosphere of 5% CO_2 at 37 °C. rBMSCs were randomly divided into three groups: control group, vector group (infected with empty vector virus). rBMSCs/ITGA5B1 group (infected with integrin alpha5beta1 virus). The ITGA5B1 expression plasmids (6 μg) together with the packaging plasmids psPax2 (8 μg), VSV-G (3 μg) and pRSV Rev (3 μg) were co-transfected into 293T cells to generate the LV-ITGA5B1 lentivirus particles by lipofectamine 2000 (Thermo Fisher Scientific). The lentiviral particles were harvested at 48 h and 72 h post-transfection, cells supernatants were centrifuged at 5000 × g for 30 min to remove cell debris, and then filtered through a Steriflip-HV 0.45 μm PVDF Filter Unit (Millipore, Billerica, MA, USA). The virus particles were concentrated by the PEG-it virus precipitation solution following the manufacturer's instructions (SBI, USA), and then the rBMSCs were transduced with empty vector and LV-ITGA5B1 lentivirus at MOI (multiplicity of infection) of 50 respectively when it cultured to 60–70 % confluence. The transduction efficiency was observed under fluorescent microscopy (CKX71, Olympus) five days post transduction.

2.4. Western blotting

To determine the protein expression of alpha5, beta1 integrins, eNOS and actived-caspase-3, total cellular proteins was extracted by incubating cells in ice-cold lysis buffer containing PMSF (Beyotime, Jiangsu, China) for 30 min on ice after five days post-transduction. The lysate was then centrifuged at 12,000 × g for 5 min. The supernatant was collected and the protein concentration was determined by BCA Protein Assay Kit (Beyotime, Jiangsu, China). Equal amounts of protein (15 μg) were separated by 10% SDS-PAGE, and then were electrotransferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA). The membranes were blocked with 5% skimmed milk at room temperature for one hour, and then incubated with the primary monoclonal anti-alpha 5, anti-beta 1 integrin antibody (Abcam, Cambridge, UK), anti-eNOS antibody (BD Biosciences, San Diego, CA, USA), anti-β-actin antibody (Beyotime, Jiangsu, China) and polyclonal anti-actived-caspase-3 (Bioworld technology, St. Louis Park, MN, USA) respectively at dilution of 1:1000 overnight at 4 °C. The membranes were washed three times for 5 min in TBST and then incubated with species-specific horseradish peroxidase-linked secondary antibodies (1:1000) for one hour and visualized using ECL kit (Beyotime, Jiangsu, China). Images were obtained and analyzed with AlphaView analysis system (ProteinSimle, USA). The expression of β-actin was used as an internal control.

2.5. Cell adhesion and anoikis assay

Coat 96-well plates with Matrigel (1:2 dilution) overnight at 4 °C. Plates were then washed with PBS for two times to remove excess matrices and chill the plates on ice. rBMSCs (5×10^5/ml) in each groups with serum-free DMEM-F12 were added to each well, spun at 400 g for 2 min, and then incubation at 37 °C for 20 min. At checking time, the plates were agitated twice for 30 s and the unattached cells were removed by gently washed with PBS. The remaining cells were fixed with 4% Paraformaldehyde (PFA) for 15 min, stained with 0.1% (m/v) crystal violet for 15 min, and washed in water. The stain was extracted from the cells with 2% SDS, and the absorbance was read by Multiskan MK3 microplate reader at 590 nm.

Anoikis was detected by CytoSelect™ 24-well Anoikis Assay kit (Cell Biolabs, Inc) according to the manufacturer's instructions. Briefly, each group of cells (1×10^6 cells/ml) were cultured in CytoSelect™ 24-Well at 37 °C with 5% CO_2 for 24 h. 1 μl of Calcein AM (500 ×) was added to each well, then incubate the plate at 37 °C for 60 min. The live cells were observed for the presence of the green Calcein AM fluorescence under fluorescent microscopy (CKX71, Olympus).

2.6. Cell viability assay

Cell viability was evaluated by CCK-8 (Cell Counting Kit, Beyotime, Jiangsu, China) according to the manufacturer's protocol. Briefly, each