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Aspirin inhibits growth and enhances cardiomyocyte differentiation of bone marrow mesenchymal stem cells

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ABSTRACT

This study aimed to examine the effects of aspirin on the growth and cardiomyocyte differentiation grade of bone marrow mesenchymal stem cells (BMMSCs). BMMSCs were divided into five differentiation groups with different concentrations of aspirin (0 mM, 0.5 mM, 1 mM, 2 mM, or 5 mM), and a undifferentiated control group. Cell growth was measured by cell proliferation, apoptosis assays and DNA cycle analysis. The differentiation grade of BMMSC-derived cardiomyocyte-like cells was examined by measuring the levels of cardiac-specific proteins with cyto-immunofluorescence staining, flow cytometry, and Western blotting. Electrophysiological analyses were performed by patch-clamp experiments and calcium transients were measured by a laser scanning confocal microscope. Cell proliferation decreased as the concentration of aspirin increased. Cell apoptosis increased with increasing aspirin concentration. DNA replication was inhibited in the high dose-aspirin group compared to the low dose- or non-aspirin groups. The number of α-myosin heavy chain (α-MHC) and cardiac troponin I (cTnI) positive cells, cardiac troponin T (cTnT) and connexin 43 (Cx43) positive rates, expression levels of Cx43, Nkx2.5, GATA4 and β1 adrenoceptor increased with increasing aspirin concentration. No sarcomeric cross-striations, spontaneous or induced beating activity or action potentials was observed in each group. Calcium transients were measured in small number cells in 2 mM aspirin group, but the features are atypical. Consequently, aspirin inhibits proliferation and survival of BMMSCs and enhances cardiomyocyte differentiation of BMMSCs.

1. Introduction

Medical or surgical treatment of ischaemic heart disease only temporarily delays disease progression without salvaging the lost cardiomyocytes. However, mature cardiomyocytes possess a limited potential to regenerate. Stem cells capable of differentiating into cardiomyocytes have shown the potential to repair damaged myocardium, resulting in a possible treatment for myocardial infarction (Karantalis and Hare, 2015). Bone marrow mesenchymal stem cells (BMMSCs) are one of the most heavily investigated multipotent stem cell types and have the attributes of convenient acquisition, high self-renewal capacity, and immune tolerance (Hare et al., 2012; Usunier et al., 2014). The effectiveness of therapy depends primarily on the survival and differentiation of BMMSCs.

Antiplatelet therapy is the cornerstone of prevention and treatment of coronary heart disease. As the earliest and most generally used antiplatelet agent, aspirin is recommended long-term for patients with coronary heart disease to control the disease (Levine et al., 2016). Recent studies have shown that aspirin affects the proliferation and differentiation of many cell types (Cao et al., 2015; Chen et al., 2014; Huang et al., 2016). However, currently there is no evidence that indicates whether aspirin influences the progress of cardiomyocyte differentiation of BMMSC.

Aspirin is essential for patients with coronary heart disease, and stem cell therapy is an excellent prospect for cardiac repair. Both aspirin and BMMSCs will probably be used together in the treatment of patients with myocardial damage. Hence, this study aims to demonstrate that aspirin has a synergistic effect on stem cell therapy for patients with coronary heart disease. Although there is an adverse effect on the proliferation of BMMSCs, more important is that aspirin potentiates the cardiomyocyte differentiation of BMMSCs.

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2. Materials and methods

2.1. Culture and identification of BMMSCs

BMMSCs were obtained from the femoral and tibial bone marrow of 4-week-old Sprague-Dawley (SD) rats (Vital River Laboratory Animal Technology, Co., Beijing, China) and isolated by density gradient centrifugation. All animal experiments conformed to the standards of experimental animal use of the People's Republic of China (GB14925-2010). Cells were plated in complete medium (low-glucose DMEM/F-12 media containing 10% fetal bovine serum and 100 U/ml penicillin/streptomycin; Thermo Fisher Scientific, Inc., Waltham, MA, USA), and incubated at 37 °C and 5% CO_2 in a humidified atmosphere. The culture medium was replaced every 3 days. BMMSCs were passaged at a ratio of 1:2 with 0.25% Trypsin/EDTA until 90% coverage.

The third-generation BMMSCs were characterized by surface markers using flow cytometry, and by induced differentiation into adipocytes and osteocytes. Cells were harvested by trypsinization and resuspended with phosphate-buffered saline (PBS), then stained by antibodies CD29 (1:100, Biolegend, San Diego, CA. catalogue number: 102205, clone number: HMβ1-1), CD44 (1:100, Biolegend, 203906, OX-49), CD31 (1:100, Biorbyt, orb221348), CD19 (1:100, Biorbyt, orb1699), CD34 (1:100, Biorbyt, orb56005, ICO-115), CD45 (1:100, Biolegend, 202207, OX-1). The expression of cell surface epitopes was analysed with flow cytometry.

Cells plated in 6-well plates at a density of 1×10^4 cells/cm^2 were incubated for 21 days in adipogenic differentiation medium (Cyagen, Suzhou, China) and osteogenic differentiation medium (Cyagen), respectively. Adipogenesis was assessed by incubating cells with Oil Red O solution and osteogenesis was visualized by Alizarin red staining solution.

2.2. Inducing cardiomyocyte differentiation from BMMSCs and aspirin treatment

Cells were plated at a density of 1×10^4 cells/cm^2, then divided into: 1) Non-aspirin group: BMMCSs were incubated in differentiation medium (complete medium supplemented with 5 μmol/l 5-azacytidine) for the first 24 h, then switched to complete medium (without 5-azacytidine) containing 0 mM aspirin for the next 28 days; 2) Aspirin group containing 4 subgroups according to the concentration of aspirin: BMMSCs were maintained in differentiation medium for the first 24 h, then treated with complete medium (without 5-azacytidine) containing aspirin in different concentrations (0.5 mM, 1 mM, 2 mM, 5 mM) for the next 28 days; 3) Control group: BMMSCs were cultured only in complete medium. The buffering agent HEPES (25 mM) was added to maintain the pH of the cell culture media. The effects of aspirin on cell survival and proliferation were evaluated for the first 7 days. The effects of aspirin on myocardial differentiation were examined until the end of incubation. Culture media was replaced every 3 days.

2.3. Proliferation of induced BMMSCs treated with aspirin

To conduct an assessment of the cytotoxicity and proliferation inhibition of aspirin, the proliferation of each group was measured by AQueous One Solution Cell Proliferation Assay. After plating in 96-well plates at a density of 1×10^4 cells/cm^2, cells were treated with or without 5-azacytidine on day 1 and One Solution Reagent (Promega Corporation, WI, USA) was added to the culture on days 2, 4, 6, and 8, followed by incubation for 4 h at 37 °C, avoiding exposure to light. Absorbance at 490 nm was read using a Synergy 4 microplate reader (Bio Tek Instruments, Inc., Winooski, VT, USA). A curve was then established to reflect the number of living cells.

2.4. DNA cycle analysis for the measurement of cell proliferation

Cell cycle analysis was performed to investigate whether aspirin regulates cell cycle progression. BMMSCs plated in 6-well plates at a density of 1×10^4 cells/cm^2 were harvested by trypsinization in the logarithmic phase (day 3) at 1×10^6/ml from each group, fixed with 75% ethanol overnight at − 20 °C, then resuspended in PI/RNase Staining Buffer Solution (BD Pharmingen) and incubated for 15 min at room temperature. The DNA cycle was detected by flow cytometry, and cell cycle data were analysed with a ModFit LT 3.1 README FILE (Verity Software House, Inc., Topsham, ME, USA).

2.5. The effect of aspirin on BMMSC apoptosis detected by flow cytometry

To evaluate whether aspirin induced apoptosis in BMMSCs, cells plated in 6-well plates at a density of 1×10^4 cells/cm^2 in the biomass logarithmic phase of the three groups were harvested and resuspended at 1×10^6/ml with binding buffer. BMMSCs were incubated using an FITC annexin V apoptosis detection kit (BD Pharmingen, Inc., San Jose, CA, USA), followed by staining with FITC annexin V/propidium iodide in the dark for 15 min. Fluorescence was read and analysed using an EPICS XL Flow Cytometer and EXPO32 ADC software (Beckman Coulter, Inc., Brea, CA, USA).

2.6. Immunofluorescent staining for BMMSC differentiation

Immunofluorescence staining of α-MHC and cTnI (cardiac-specific proteins) was performed at day 28 to identify whether aspirin enhanced cardiomyocyte differentiation of BMMSCs. Cells were fixed with 4% paraformaldehyde for 15 min at 4 °C, permeabilized with 0.5% Triton-X 100 for 10 min, and blocked with 1% bovine serum albumin (BSA) for 30 min at room temperature, then incubated with primary anti-cardiac myosin heavy chain (α-MHC) antibody (1:200, Abcam, Cambridge, USA. ab 50967, BA-G5) and cardiac troponin I (cTnI) antibody (1:200, Abcam, ab 47003) overnight at 4 °C, followed by staining with FITC-conjugated secondary antibody (1:1000, Abcam, ab 6785; ab 6717) for 1 h at room temperature out of direct light. Cell nuclei were stained by 4', 6-diamidino-2-phenylindole (DAPI, 5 μg/ml) for 5 min. The slides were imaged using a Nikon Eclipse 600 fluorescence microscope (Nikon Instruments Inc., Tokyo, Japan). High-resolution images of cells in the 2 mM aspirin group were scanned using a laser scanning confocal microscope (Leica, Mannheim, Germany).

2.7. Flow cytometry detection of BMMSC differentiation rate

Flow cytometry analysis was performed to compare the cardiomyocyte differentiation rate of BMMSCs in the different groups. Fixation buffer (Shanghai Yeasen Biotechnology Co., Shanghai, China) was added to harvested cells for 10 min at 25 °C. Cells were washed and resuspended at 1×10^6/ml with permeabilization buffer (Shanghai Yeasen Biotech Co.) for 10 min at 25 °C, then incubated in PBS with cardiac troponin T (cTnT) antibody (1:200, Abcam, ab 8295, 1C11) and connexin-43 (Cx43) antibody (1:200, Abcam, ab 79010, 4E6.2) for 1 h. The primary antibodies were removed, and cells were stained with FITC-conjugated secondary antibody (1:500, BD Biosciences, 554001) for 30 min. Fluorescence values were read using a flow cytometer.

2.8. Western blotting for cardiac specific proteins

The expression of the cardiac specific proteins Cx43, Nkx2.5, GATA4 and β1 adrenoceptor were determined using Western blotting. Cells were rinsed and lysed in lysis buffer, followed by determination of the protein concentration using a BCA Protein Assay kit (Beyotime Biotechnology, Jiangsu, China). Equal amounts of protein were electrophoresed and transferred to membranes, then blocked with skim milk powder solution for 2 h at room temperature. The membranes