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Laboratory Studies

Role and mechanism of microRNA-21 in H_2O_2-induced apoptosis in bone marrow mesenchymal stem cells

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ABSTRACT

microRNA-21 (miR-21) contributes to anti-apoptosis, proliferation and migration in many cells, but its role in inhibiting apoptosis in bone marrow mesenchymal stem cells (BMSC) remains unclear. The aim of this study was to determine the role of miR-21 in H_2O_2-induced BMSC apoptosis. We used quantitative real time-polymerase chain reaction (RT-PCR) to demonstrate the level of miR-21 after treatment of BMSC with H_2O_2. BMSC apoptosis was induced by different concentrations of H_2O_2 and was decreased in miR-21-upregulated cells. The expression of PTEN, a functional target gene of miR-21 in BMSC, was regulated by miR-21. The RT-PCR results indicated that miR-21 was significantly up-regulated, and western blot analysis indicated that Bcl-2 was up-regulated, whereas the apoptosis-related genes caspase 3/9 and Bax were down-regulated in miR-21-up-regulated cells. The miR-21-up-regulated cells had significantly enhanced Akt phosphorylation, as measured by western blot analysis. LY294002, an inhibitor of Akt activation, abolished the protective effects of miR-21-up-regulated cells. These results suggest that miR-21 contributes to inhibition of apoptosis in BMSC by down-regulating PTEN, potentially via the PI3K/Akt pathway.

1. Introduction

The number of patients with spinal cord injury (SCI) resulting from motor vehicle accidents, falls or other high-energy injuries increases every year. SCI often leads not only to lifelong disability, but also to physical and economic burden on the patient's family and society. SCI is one of the most common and devastating injuries plaguing spine surgeons and neurosurgeons [1]. The treatment of SCI remains one of the greatest challenges in basic science and clinical research. Although many novel therapies have been explored, all current therapies have demonstrated limited efficacy. In recent years, stem cell transplantation treatment has been a research area of intense interest [2], but there are still many problems with this treatment, including poor survival and limited proliferation of the transplanted cells and the formation of glial scars [3].

microRNA (miR) constitute a class of small, endogenous, non-coding, single-stranded RNA consisting of 20 to 23 nucleotides that exist widely in all types of animals and plants. These RNA are involved in almost every biological process, including cellular differentiation and proliferation, metabolism and apoptosis [4,5]. miR act in the post-transcriptional regulation of small molecules and thereby affect the synthesis of the corresponding protein by binding the 3′-untranslated region (UTR) of the mRNA of the target gene [6,7]. Microarrays performed after SCI identified 60 miR that were up-regulated or down-regulated at moderate to high levels compared with the levels observed in normal spinal cord tissue, and many of these miR are involved in pathophysiological events secondary to SCI, including inflammation, oxidation, and apoptosis [8]. miR-21 plays an important role in limiting secondary cell death following SCI through its regulation of apoptosis-related genes [9]. The beneficial effects of exercise act on miR-21 and its target gene PTEN, which contribute to the functional regulation of apoptosis after SCI [10]. In many solid tumors, miR-21 is closely related to the biological behavior of tumor cells, including proliferation, migration, and invasion [11]. A previous study confirmed that miR-21 protects mouse cardiac myocytes from hydrogen peroxide (H_2O_2)-induced injury [12]. However, the effects of miR-21 represent a new layer of gene expression regulation at the translational level, and the effects of H_2O_2 on miR-21 expression in bone marrow mesenchymal stem cells (BMSC) are unknown. In addition, the roles of miR-21 in H_2O_2-mediated gene regulation and its biological functions in BMSC remain to be elucidated.

PTEN was the first tumor suppressor gene to be discovered; it is closely associated with carcinoma and has bispecific phosphatase activity [13]. The PTEN gene inhibits Akt activation (phosphorylation), which plays an important role in growth, apoptosis, adhesion, migration, and invasion [14,15]. The silencing of PTEN can

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promote cell proliferation [16]. Our previous study indicated that PTEN gene silencing inhibits cell apoptosis and enhance cell viability, but its pathogenesis is not clear [17]. Interestingly, through bioinformatics approaches, we found that PTEN is a theoretical target gene of miR-21.

In this study, we provide, to our knowledge, the first evidence that miR-21 may protect BMSC against H_2O_2-induced apoptosis through the PTEN/Akt pathway and enhance the resistance of BMSC to oxidative stress. This suggests miR-21 may possess the ability to protect BMSC from oxidative injury, and may be relevant to stem cell treatment of SCI.

2. Materials and methods

2.1. Cell culture and grouping of BMSC

The cell strain of BMSC used in this study was purchased from the Cyagen Corporation (Guangzhou, China) as previously described [17]. The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM)/F12 (Hyclone, CA, USA) supplemented with 10% fetal bovine serum (Gibco, CA, USA), 100 IU/ml penicillin and 100 mg/ml streptomycin and incubated at 37°C in a humidified chamber supplemented with 5% CO_2. Once the cells reached a confluence of approximately 80%, the cells were harvested with a 0.25% trypsin-0.02% ethylenediaminetetraacetic acid (EDTA) solution and passaged. The BMSC were divided into three groups: the blank control group (normal), the negative control group, and the miR-21-up-regulated group (miR-21-up). The cells in the blank control group were normally cultured without any handling. The cells in the negative control group were transfected with a scrambled sequence, whereas the cells in the miR-21-up group were transfected with miR-21-up lentivirus to upregulate the expression of miR-21.

2.2. Transfection of BMSC with miR-21-up lentivirus

The BMSC were plated into six-well plates and grown to 30–50% confluence after 24 hours of incubation and were then transfected with the miR-21-up lentivirus (LV-rno-miR-21, 9132-1) and negative control (LVCON145) at a final multiplicity of infection of 40. The cells were then diluted in DMEM/F12 without serum (GeneChem, Shanghai, China). The efficiencies of miR-21-up and the negative control were tested by quantitative real time polymerase chain reaction (qRT-PCR) and western blotting.

2.3. RNA isolation and qRT-PCR

Total RNA was extracted using the Trizol reagent according to the manufacturer's instructions (Invitrogen, USA). The PCR reverse primer for miR-21 was provided by the GenePharma Corporation (China), and the specific forward and reverse primers and probe sequences were 5′-TCGCCCGTAGCTTATCAGACT-3′, 5′-CAGAG CAGGGTCCGAGGTA-3′ and 5′-CGCTCTGGACCCGACTCAACA-3′. The miRNA expression data were normalized to the expression of U6 snRNA. qRT-PCR for miR-21 was performed using cDNA generated from 1 μg of total RNA according to the manufacturer's protocol. The RT-PCR reactions were performed using a Hairpin-it miRNA RT-PCR Quantitation Kit (GenePharma), and the specific products were detected and analyzed using a Roche LightCycler 480 Detection System (Roche, Switzerland).

2.4. Cell viability assay

The cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [18]. Following pretreatment with different concentrations of H_2O_2 for 24 hours after the removal of medium, 10 μl of a 5 mg/ml MTT solution was added to each well, and the plates were incubated at 37°C for 4 hours. Approximately 150 μl of DMSO was added to each well to dissolve the formazan crystals, and the absorbance was measured at 490/570 nm. Non-treated BMSC were considered controls, and the results for the treated groups are expressed as percentages of the control. The results were obtained from three independent experiments performed in quadruplicate.

2.5. Analysis of cell apoptosis

Apoptosis was determined by detecting phosphatidylserine exposure on the cellular plasma membranes using the fluorescent dye Annexin V-FITC/propidium iodide (PI) and the Annexin V-allophycocyanin (APC)/7-amino-actinomycin D (7AAD) Apoptosis Detection Kit according to the manufacturer's protocols (KeyGEN, China). In brief, the cells were harvested, washed twice in ice-cold phosphate buffered saline (PBS) resuspended in 500 μl of binding buffer, incubated with 5 μl of Annexin V-FITC (APC) and 5 μl of PI (7AAD) solution for 15 minutes at room temperature in the dark and then immediately analyzed by bivariate flow cytometry using a FACScan-LSR equipped with CellQuest (BD Biosciences, Franklin Lakes, NJ, USA) software. Approximately 5×10^5 cells were analyzed in each of the samples.

2.6. Effect of H_2O_2 on miR-21 expression

The cultured rat BMSC were treated with either vehicle or different concentrations of H_2O_2 (0–150 μmol/L) for 6 hours. RNA was then isolated and determined as described previously.

2.7. Observation of the morphology of apoptotic cells

The morphology and number of BMSC were directly observed using an inverted phase contrast microscope. The cells were cultured in 24-well plates, and after apoptosis, the cells were fixed for 10 minutes, stained with 125 μl of a Hoechst33258 staining solution for 5 minutes, washed twice with PBS, dropped with an anti-fading solution and then imaged using a camera with a fluorescence microscope.

2.8. Western blot analysis

>Equal amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) on a 12% running gel and 5% stacking gel, and the protein blots were then transferred to a polyvinylidene fluoride (PVDF) membrane (0.45 μm; Millipore; Merck, Darmstadt, Germany). The membrane was blocked with 5% nonfat milk in a mixture of tris-buffered saline and tween 20 (TBST) for 2 hours and incubated with a primary antibody against PTEN, Akt, p-Akt (rabbit, 1:1000; Cell Signaling Technology, Danvers, MA, USA), caspase 3, caspase 8, caspase 9 (rabbit, 1:5000, Abcam, Cambridge, UK), Bcl-2, Bax (rabbit, 1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and GAPDH (rabbit, 1:2000, Santa Cruz Biotechnology) in TBST containing 5% bovine serum albumin overnight at 4°C. After washing three times with TBST, the membrane was incubated at room temperature for 2 hours with horseradish peroxidase-conjugated secondary antibody (anti-rabbit, 1:2000, Cell Signaling Technology) diluted with TBST. The detected protein signals were visualized using an enhanced chemiluminescence (ECL) system western blot kit (Thermo Fisher Scientific, Waltham, MA, USA).

2.9. Statistical analysis

All analyses were performed with one-way analysis of variance using the Statistical Package for the Social Sciences version 13.0