Original Article
miR-181a promotes apoptosis in contusion spinal cord injury rats through targeting Bcl-2

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Received August 23, 2016; Accepted November 6, 2016; Epub February 15, 2017; Published February 28, 2017

Abstract: Many studies demonstrated that apoptosis would aggravate the secondary injury to the spinal cord and reduced apoptosis can improve the functional recovery after spinal cord injury (SCI). In many animal models and cell types, microRNAs (miRNA) have been implicated as effectors of SCI. However, little is known about the role of microRNAs in the regulation of apoptosis and molecular mechanism in SCI. This paper aims to explore the correlation between reactive oxygen species (ROS)-induced cell apoptosis and dysregulation of microRNAs after SCI. We analyzed six miRNA expression patterns at 1, 3 and 7 days following rat SCI using reverse transcription quantitative PCR (RT-qPCR). Because miR-181a was one of the six miRNAs being most significantly upregulated, we investigated its function. The immortalized murine BV-2 cells were treated with H$_2$O$_2$. The results demonstrated that H$_2$O$_2$ significantly enhanced ROS production, reduced cell viability and induced BV-2 cell apoptosis. We also found that H$_2$O$_2$ significantly enhanced miR-181a expression in a dose- and time-dependent manner. Following miR-181a silencing, H$_2$O$_2$-induced cell viability and apoptosis were rescued in BV-2 cells, and the levels of Caspase 3,8,9 were enhanced in BV-2 cells treated with H$_2$O$_2$. Furthermore, Bcl-2 was identified as a direct target of miR-181 a using a Luciferase reporter assay, RT-qPCR and Western blot analysis. Based on previous studies, it was predicted that miR-181a contributed to apoptosis in murine BV-2 cells by regulating the expression of Bcl-2. This proposes a therapeutic target for enhancing neural cell functional recovery after SCI.

Keywords: Spinal cord injury, BV-2, apoptosis, miR-181a, Bcl-2

Introduction

Spinal cord injury (SCI) is one of the most common and devastating injuries observed in spine and neurosurgery departments [1]. In our modern society, the incidence of SCI is strongly related to traffic accidents, sports accidents, overburden and falling from a height. It can cause permanent disabilities such as paralysis and loss of movement or sensation, and bring heavy burden to society and family. Although many therapies have been explored, all current therapies have demonstrated limited efficacy.

Apoptosis is the main approach of secondary injury after SCI [2, 3]. However, the precise mechanism of such apoptosis is not fully understood. Much of the early data regarding apoptotic death was confined to the study of neurons but it occurs also in oligodendrocytes and microglial cells [4]. To explore the pathologic factors after SCI, the immortalized murine BV-2 cell line was applied as it is reported to share many characteristics with primary microglia. Previous studies demonstrated that changes in gene expression after SCI may have a strong impact on the pathology [5, 6]. Importantly, many miRNAs have extensive expression in the central nervous system and play important roles in development and function [7].

MiRNAs are endogenous, non-coding ~22 nt RNA molecules that negatively regulate gene expression at posttranscriptional level [8, 9]. And, kinds of miRNAs have been confirmed to be associated with apoptotic pathways. For example, MiR-125b functions as an anti-apoptotic factor by inhibiting the expression of anti-apoptotic gene Bcl-2 [10]. MiR-34a induces apoptosis in the human glioma cell line through enhanced ROS production and NOX2 expression [11]. The miR Let-7a may function as a pro-apoptotic factor by its effects on the anti-apoptotic genes RAS and MYC [12, 13]. A study from Wang L et al. demonstrated that H$_2$O$_2$-induced up-regulation of miR-181a significantly contrib-
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uted to apoptosis of cardiomyocytes by maintaining hexokinase II (HKII) expression [14]. However, little research has been conducted on the expression of miR-181a after SCI.

In the present study, we detected the expression of six miRNAs associated with apoptosis in a rat moderate contusion SCI model. We observed that miR-181a was the most dysregulated microRNA after SCI. Moreover, we identified miR-181a as a Bcl-2-targeting miRNA and demonstrated that its expression increased during ROS-induced apoptosis of BV-2. We further examined the potential of anti-miR-181a as an anti-apoptotic agent for BV-2 exposed to ROS in vitro.

Materials and methods

Animals

Adult male Sprague-Dawley (SD) rats, weighing 180-220 g, were provided by the Center of Experimental Animals, Henan Provincial People’s Hospital. All animal care, breeding, and testing procedures were approved by the Laboratory Animal Users Committee at Henan Provincial People’s Hospital, Zhengzhou, China. All animals were housed in individual cages in a temperature-and light cycle-controlled environment with free access to food and water.

Establishment of contusion SCI model

Rats were anesthetized with an intraperitoneal injection of 10% chloral hydrate (3 mg/kg). A laminectomy was performed at thoracic vertebra level 10 (T10). Moderate contusion injury was induced using a modified Allen’s weight drop apparatus (8 g weight at a vertical height of 40 mm, 8 g × 40 mm) on the exposed dura of the spinal cord. Sham injured animals were only subjected to laminectomy [15].

Cell culture and drug treatments

The immortalized murine BV-2 cell line was purchased from the Chinese Academy of Medical Science and cultured in DMEM/F12 (HyClone, Waltham, MA, USA) supplemented with 10% fetal bovine serum (HyClone), 100 U ml⁻¹ penicillin and streptomycin in 25-cm² culture flasks at 37°C in a humidified atmosphere with 5% CO₂. Cells were treated with the following drugs. H₂O₂ (30% w/w solution; Sigma, St Louis, MO, USA) was administered to the cells as a 100 mM solution in phosphate-buffered saline (PBS). N-acetyl-L-cysteine (Sigma) was dissolved in water.

RNA extraction and reverse transcription quantitative PCR

Total RNA was isolated using TRIzol (Invitrogen, CA) and miRNaseasy mini kit (Qiagen, West Sussex, UK) according to manufacturer’s instructions. Total RNA from each sample was reverse-transcribed to cDNA using the PrimeScript RT reagent Kit (TaKaRa, Tokyo, Japan) and qRT-PCR was performed using the SYBR Premix Ex Taq (TaKaRa, Tokyo, Japan) and miRNA-specific primers for miR-181a (Ribobio, Guangzhou, China). The relative microRNA levels were normalized to U6 expression for each sample. Analysis of gene expression was performed by the 2⁻ΔΔCt method.

MTT cell viability assay

BV-2 cells were seeded in 96-well culture plates with 1×10⁴ cells/well, and incubated at 37°C with 5% CO₂. After treating with 100 μM of H₂O₂, MTT assay (Amresco, Solon, USA) was performed. Briefly, 20 μL of MTT solution (5 mg/ml) was added to each well, and the cells were continuously incubated for 4 h. Formazan crystals were then dissolved in 150 μL DMSO. The optical density (OD) of the wells was measured with a microplate reader (BioTek, Richmond, USA) at 490 nm.

Apoptosis assay

To detect the effects of miR-181a on BV-2 cell apoptosis, the cells (50-60% confluent) were transfected with miR-181a mimics, inhibitor or negative control. After treatment, the cells were washed with 1 × PBS for three times. Then, an Annexin-V FITC-PI Apoptosis Kit (Invitrogen) was applied to determine the apoptotic rate by flow cytometry. This assay employed fluorescein-labeled Annexin-V in concert with propidium iodide to detect the cells undergoing apoptosis.

Transfection

The BV-2 cells were plated into six-well plates and grown to 30-50% confluence after 24 hours of incubation and were then transfected with miRNA mimics, miRNA inhibitor and negative control at a final multiplicity of infection of 10 using siLentFect™ Lipid reagent (Life
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Science Research). The cells were then diluted in DMEM/F12 without serum (GeneChem, Shanghai, China). After 4 h of incubation in a CO₂ incubator at 37°C, the medium was changed to 10% FBS containing DMEM. The efficiencies of miRNA mimics, miRNA inhibitor and negative control were tested by quantitative real time polymerase chain reaction (qRT-PCR).

Dihydroethidium staining

Cells cultured on six-well chamber slides were washed with PBS three times for 5 min per wash, and then the slides were incubated with ROS Fluorescent Probe-dihydroethidium (Vigorous Biotechnology Beijing Co., Ltd, Beijing, China) in serum-free DMEM F12 medium for 30 min at 37°C in darkness, fixed in 4% paraformaldehyde for 30 min at room temperature (RT). The slides were washed again and mounted. Immunofluorescence images were captured by fluorescence microscopy [16].

Luciferase reporter assay

Dual luciferase assays were conducted in a 24 well plate format. pGL3-Bcl-2 3'UTR report/ pGL3-Bcl-2 3'UTR Mutant report+TK100 Renilla report were transfected into 70% confluent HEK293 cells, along with miR-181a mimic, miR-181a inhibitor or each control. After 48-h transfection, firefly and renillaluciferase were quantified sequentially using the Dual Luciferase Assay kit (Promega, USA) following the manufacturer’s recommendations.

Western blot analysis

Protein extracts from BV-2 cells were subjected to 10% SDS-PAGE and subsequently transferred to a PVDF membrane. The membrane was blocked with 5% (w/v) nonfat milk and incubated sequentially with the primary antibodies against caspase 3, caspase 8, cas-pase 9 (rabbit, 1:5000, Abcam, Cambridge, UK), Bcl-2 (rabbit, 1:1000, Santa Cruz Biotechnology) in TBST containing 5% bovine serum albumin overnight at 4°C. Anti-β-actin antibody was used as an internal control. After washing three times with TBST, the membrane was incubated at room temperature for 2 hours with horse-radish 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).

Statistical analysis

Statistical analyses were performed with SPSS 13.0 software. The results were evaluated by χ² test and the other data were evaluated by Student’s t-test and expressed as the mean ± SD from three independent experiments. A

Figure 1. The expression of the selected six miRNAs at 1, 3 and 7 days after spinal cord injury (SCI) was quantified by qRT-PCR analysis. Relative expression indicates changes compared with the sham group at 1, 3 and 7 days after SCI. Data represent the means ± SEM. *P<0.05; **P<0.01 vs. Control.
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P-value of less than 0.05 was considered statistically significant.

Results

Expression of miRNAs in injured spinal cord

We chose 6 recognized microRNAs that play an important role in the apoptosis and analyzed their expression in contusion SCI model using quantitative real-time polymerase chain reaction (qRT-PCR). These data confirmed that miR-34, Let-7, miR-29, miR-181a and miR-125b were over-expressed compared with the sham 1 day group, whereas the expression of miR-206 was no significant change (Figure 1A-F). Among them, miR-181a has been shown to have the greatest change in our SCI rat model, therefore, we focused on miR-181a for further study.

H₂O₂ induced cell death of BV-2 and increased miR-181a expression

As SCI was usually accompanied by significant ROS production in microglia cells, an oxidative stress model was established in vitro. After treating BV-2 cells with 100 μM H₂O₂ for 10 hours, Dihydroethidium staining showed that H₂O₂ significantly enhanced ROS production (Figure 2A). And, BV-2 cells were treated with 100 μM H₂O₂ for 12, 24 and 48 h, and then the cells were analyzed by MTT Assay, as shown in Figure 2B. H₂O₂ significantly reduced cell viability of BV-2 cells in a time-dependent manner.

To detect whether the reduction of cell viability was associated with cell apoptosis, flow cytometry assay was applied to detect the apoptotic rate of BV-2 cells treated with H₂O₂. As shown in Figure 2C, compared with the control group, apoptosis was markedly increased in BV-2 cells after treating with 100 μM H₂O₂ for 24 h.

The level of miR-181a was determined by real-time RT-PCR after treating with different concentrations of H₂O₂ in BV-2 cells for 2, 4, 8, 16 and 24 hours. Data are shown as means ± SEM, n=3 independent experiments, **P<0.01 vs. Control, ***P<0.01 vs. H₂O₂+NAC.

Figure 2. H₂O₂ induced miR-181a expression in murine BV-2 cells. A. Murine BV-2 cells were exposed to 100 μM H₂O₂ for 10 h. ROS production was determined using dihydroethidium staining and flow cytometry. B. Murine BV-2 cells were exposed to 100 μM H₂O₂ for 24, 36 and 48 h. The growth of Murine BV-2 cells was measured using an MTT assay. C. Murine BV-2 cells apoptosis after treatment with 100 μM H₂O₂ for 24 hours was assessed. The cells were stained with Annexin V-FITC and PI and subjected to flow cytometric analysis. D and E. The level of miR-181a was determined by real-time RT-PCR after treating with different concentrations of H₂O₂ in BV-2 cells for 2, 4, 8, 16 and 24 hours. Data are shown as means ± SEM, n=3 independent experiments, **P<0.01 vs. Control, ***P<0.01 vs. H₂O₂+NAC.
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**Figure 3.** Inhibition of miR-181a alleviates H$_2$O$_2$-induced apoptosis of BV-2 cells. A. The expression of miR-181a after treatment with miR-181a inhibitor or negative control (n=6). B. The growth of Murine BV-2 cells was measured using an MTT assay after transfection with miR-181a inhibitor or negative control. Knock down of miR-181a mitigates the reduction of cell viability induced by H$_2$O$_2$ in BV-2 cells. C. The apoptosis of Murine BV-2 cells was measured after transfection with miR-181a inhibitor or negative control. Knockdown of miR-181a alleviates H$_2$O$_2$-induced apoptosis of BV-2 cells. D. The expression of caspases 3, 8, and 9 is assessed at 24 h after transfection with miR-181a inhibitor, or inhibitor control. E. Activity of caspases 3, 8, and 9 is assessed at 24 h after transfection with miR-181a inhibitor, or negative control. Data are shown as mean ± SEM, n=3 independent experiments, *P<0.05 vs. control; **P<0.01 vs. control; ##P<0.01 vs. H$_2$O$_2$+miR-181a inhibitor.

apoptosis in BV-2 cells by regulating the expression of miR-181a.

**MiR-181a decreased H$_2$O$_2$-induced apoptosis of BV-2 cells**

To confirm the role of miR-181a in the process of apoptosis, we transfected miR-181a inhibitor into BV-2 cells (Figure 3A), followed by treatment with 100 μM H$_2$O$_2$ for 24 hours. Our data showed that miR-181a restored the reduction of cell viability induced by H$_2$O$_2$ (Figure 3B). And, knockdown of miR-181a alleviated H$_2$O$_2$-induced apoptosis of BV-2 cells (Figure 3C). Both caspase-dependent and-independent pathways are known to be involved in the process of apoptosis. We tested whether the apoptosis of BV-2 cells caused by H$_2$O$_2$ was associated with the activation of caspase-3/8/9. To address this question, Western blot were used to examine the expression changes of caspase-3 8, and 9 proteins. As shown in Figure 3D, silencing miR-181a decreased the expression of caspases 3, 8, and 9. Moreover, we found that knockdown of miR-181a decreased the caspase-3, 8 and 9 activity in BV-2 cells (Figure 3E), indicating that the elevation of miR-181a is involved in the process of H$_2$O$_2$-induced apoptosis in BV-2 cells.

**MiR-181a mediated H$_2$O$_2$-induced apoptosis by decreasing Bcl-2 activity**

To examine whether miR-181a directly targets Bcl-2, we conducted a luciferase assay (Figure 4A). The luciferase assay used in the present study is to evaluate the effect of miRNA-dependent post-transcriptional regulation of target
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Figure 4. miR-181a directly binds and downregulates Bcl-2. A. Schema of the firefly luciferase reporter constructed for the Bcl-2, indicating the interaction sites between miR-181a and the 3'-UTRs of the Bcl-2. B. The expression of miR-181a after treatment with miR-181a mimic or mimic NC (n=6). C. Murine BV-2 cells were co-transfected with firefly luciferase constructs containing the Bcl-2 wild-type or mutated 3'-UTRs and miR-181a mimic, mimic NC, miR-181a inhibitor or inhibitor NC, as indicated (n=6). D and E. Protein expression of Bcl-2 after treatment with miR-181a mimic or miR-181a inhibitor (n=6). All data are shown as mean ± SD, results of three independent experiments. **P<0.01.

genes. We transfected BV-2 cells with miR-181a mimic in which miR-181a expression was significantly increased (Figure 4B). The results showed that overexpression of miR-181a significantly decreased the luciferase activity in pGL3-Bcl-2 3'-UTR transfected cells, whereas it had no effect on the cells transfected with pGL3-mut Bcl-2 3'-UTR (Figure 4C).

To evaluate whether miR-181a regulated Bcl-2 expression, we detected the protein expression level of Bcl-2 in miR-181a mimic or miR-181a inhibitor infected cells. Western blot analysis showed that miR-181a overexpression markedly decreased the protein level of Bcl-2 (Figure 4D), whereas miR-181a inhibition increased the protein expression of Bcl-2 (Figure 4E).

Discussion

In the present study, we demonstrated that H2O2 could inhibit the growth of BV-2 cells in a
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time-dependent manner. The H$_2$O$_2$ promoted the expression of miR-181a, then caused down-regulation of Bcl-2, and activation of caspase-3/8/9, and finally induced the apoptosis in BV-2 cells. More importantly, knockdown of miR-181a markedly protected BV-2 cells against H$_2$O$_2$-induced apoptosis, which indicates that miR-181a may be a novel target for the treatment of SCI.

SCI is a devastating consequence of spine fractures. Many genes have been reported to play important roles in the pathogenesis of SCI [17]. Existing researches show that many miRNAs have extensive expression in the central nervous system and play important roles in development and function [18-20]. For instance, miR-21 was significantly upregulated 3 days after SCI in rats and showed strong anti-apoptotic effects, thus indicating its protective role after SCI [21]. In the present study, the expression of six miRNAs (miR-34a, -29, 181a, -125b, -206 and let-7) were further confirmed by qRT-PCR analysis and miR-181a was found to have the greatest change in many injury models. Previous studies have found that the miR-181 family, especially miR-181a and miR-181b, are rich in the brain, and the abnormal expression of miR-181 family is related to many diseases of nervous system [22]. However, little attention has been paid to the effects of miR-181a in SCI.

After SCI, the microenvironment of ischemia/hypoxia and inflammation can induce the generation of reactive oxygen species (ROS), which include H$_2$O$_2$, superoxide anion and hydroxyl radicals, and induces cell apoptosis [16, 23]. DS Yu et al. reported that ROS production could enhance miR-200c expression in a dose-dependent manner and induced significant apoptosis in BV-2 cells [22]. In this study, we treated murine BV-2 cells with H$_2$O$_2$ to generate a cellular model of SCI and found that H$_2$O$_2$ reduced cell viability of BV-2 cells in a time-dependent manner and increased the apoptosis of BV-2 cells. Furthermore, H$_2$O$_2$ increased the level of miR-181a in BV-2 cells in a time- and dose-dependent manner. Thus far, little research has addressed ROS-induced miRNA alterations in murine BV-2 cells and their effects on spinal cord functional recovery [15].

Mitochondria plays an important role in apoptosis, and Bcl-2 gene family is a key factor to regulate apoptosis through mitochondrial pathway [24]. A study performed by Can-Jie Guo showed that the enzymatic activities of caspases 3, 8, and 9, the key executioners of apoptosis, increased significantly at the concentration of 100 nM miR-15b or miR-16 [25]. In our study, we found that knockdown of miR-181a reduced the expression of caspase 3, caspase 8 and caspase 9 induced by H$_2$O$_2$, which indicates that miR-181a upregulation may contribute to ROS-induced apoptosis in BV-2 cells after SCI. These data support an essential role for miR-181a in the regulation of the mitochondrial machinery by modulating the levels of caspase activation.

Recently, studies showed that individual miRNAs can regulate hundreds of genes simultaneously by targeting RISCs to mRNAs where they acted to inhibit translation or direct destructive cleavage [26]. To address the specific mechanism by which miR-181a induced apoptosis, we tested the impact of miR-181a on Bcl-2, which had been shown to be repressed by miR-181a in several cancer cells [27-29]. Bcl-2 protein can block the release of cytochrome c from mitochondria, which plays a role in anti-apoptosis [30]. Western blot analysis revealed that the overexpression of miR-181a reduced Bcl-2 protein levels, whereas the subsequent inhibition of miR-181a enhanced Bcl-2 expression, thereby demonstrating enhanced anti-apoptotic effects. These results indicate that miR-181a induced apoptosis in BV-2 cells mainly by suppressing Bcl-2 expression.

In conclusion, our data reveal that miR-181a protects BV-2 cells from H$_2$O$_2$-induced injury and apoptosis by targeting Bcl-2. This finding suggests that miR-181a is a novel protective molecule, which may potentially allow us to develop additional therapeutic strategies for the treatment of SCI.

Acknowledgements

This study was supported by the Research Projects of Basic and Cutting-edge Technology of Henan Province (No.122300410195).

Disclosure of conflict of interest

None.

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