MicroRNA-34a induces apoptosis in PC12 cells by reducing B-cell lymphoma 2 and sirtuin-1 expression

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Received September 6, 2014; Accepted July 3, 2015
DOI: 10.3892/mmr.2015.4185

Abstract. MicroRNA-34a (miR-34a) is a direct target of p53 and was reported to induce cell cycle arrest, apoptosis and senescence. Inhibition of the NAD-dependent deacetylase sirtuin-1 (SIRT1) by miR-34a leads to an increase in acetylated p53, which promotes cell apoptosis. B-cell lymphoma 2 (Bcl-2) is also involved in apoptosis, and was originally characterized with respect to its role in controlling outer mitochondrial membrane integrity. The effect of miR-34a in PC12 cells has not yet been reported. In the present study, it was hypothesized that Bcl-2 and SIRT1 may be critical downstream targets of miR-34a that participate in apoptosis induction. miR-34a mimics and inhibitors were transfected into PC12 cells, and the apoptosis and proliferation rates were compared between groups. It was demonstrated that induction of miR-34a promotes apoptosis and senescence, inhibits proliferation, and leads to marked alterations in SIRT1, Bcl-12 and acetyl (ac)-p53 expression. These data indicate that miR-34a may be important in neuropathy.

Introduction

MicroRNAs (miRs) are small, 19-25 nucleotide long non-coding RNAs that function as critical regulators of gene expression. They bind to the 3'-untranslated region (UTR) of target genes and, along with other accessory proteins, form an RNA-induced silencing complex that is responsible for promoting mRNA degradation and inhibiting mRNA translation (1). Increasing evidence indicates that miRNAs are essential in a number of biological processes, including development, apoptosis, cell proliferation, differentiation, disease survival and cell death (2-4). Aberrantly expressed miRNAs are also associated with several neurological diseases, such as Parkinson's disease, dementia and glioma (5-8). Current research has revealed that ectopic overexpression of microRNA-34a (miR-34a) can induce cell cycle arrest, apoptosis and senescence to inhibit cancer recurrence, migration and metastasis (9,10). Multiple studies have also indicated that miR-34a regulates a variety of target mRNAs, including cyclin-dependent kinase 4/6 (CDK4/6), E2F transcription factor3 (E2F3), Cyclin E2, B-cell lymphoma 2 (Bcl-2) and NAD-dependent deacetylase sirtuin-1 (SIRT1) (11,12). TP53 is one of the most common apoptosis-related genes in mammalian cells, and its gene product p53, activates the transcription of a set of miRNAs, including members of the miR-34 family (13). Bcl-2 is another apoptosis-related gene. Bcl-2 family members were originally characterized with respect to their roles in regulating apoptosis through complex interactions that dictate the integrity of the outer mitochondrial membrane. As an NAD-induced deacetylase, SIRT1 is a transcriptional regulator and can inhibit the expression of pro-apoptotic proteins (14). SIRT1 regulates p53-dependent apoptosis by deacetylating and destabilizing p53. It has been confirmed that SIRT1 mediates miR-34a-induced apoptosis by regulating p53 activity. A positive feedback loop has been identified, in which p53 induces expression of miR-34a, suppressing SIRT1 and increasing p53 activity (15). PC12 cells are derived from rat adrenal medulla pheochromocytoma, and are widely utilized in in vitro studies of neurological diseases. However, thus far, there have been no experimental studies of the effect of miR-34a in PC12 cells.

It was hypothesized that Bcl-2 and SIRT1 may be critical downstream targets of miR-34a that participate in cellular apoptosis. In the present study, miR-34a mimics or inhibitors were transfected into PC12 cells, and the apoptosis and proliferation rates were measured. The aim of the present study was to establish whether miR-34a-induced PC12 cell apoptosis occurs via suppression of SIRT1 and Bcl-2.

Materials and methods

Cell culture. PC12 cells (obtained from the Biomedical Laboratory of Xinjiang Medical University, Ürümqi, China)
were cultured in RPMI medium (GE Healthcare, Logan, UT, USA) containing 10% horse serum (Hangzhou Sijiqing Biological Engineering Materials Co., Hangzhou, China) and 5% fetal bovine serum (Hangzhou Sijiqing Biological Engineering Materials Co.) in a CO₂ humidified incubator at 37°C. Transfection was performed using Lipofectamine 2000 (Invitrogen Life Technologies, Carlsbad, CA, USA) kit according to the manufacturer's instructions. The cells were divided into the following groups: Negative control group (control group), 100 nM miR-34a mimic (miR-34a mimic group) and 100 nM miR-34a inhibitor (miR-34a inhibitor group). The miR-34a mimic and inhibitor were obtained from Shanghai Genechem Co., Ltd. (Shanghai, China).

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. PC12 cells were seeded into 96-well plates at a density of 4x10³ cells/well. The effect of miR-34a on cell growth and viability was determined by an MTT assay. After transfection (24, 48 or 72 h) with either miR-34a mimic or miR-34a inhibitor, cells were incubated with MTT (5 mg/ml) in phosphate-buffered saline (PBS) for 4 h, and then lysed with 50% N,N dimethylformamide and 10% SDS for an additional 3 h at 37°C. The absorbance was measured at 570 nm using an ELISA reader (DG-3022; Nanjing Huangdong Electronic Information & Technology Co., Ltd, Nanjing, China). Samples were plated in triplicate, and the average value for each group was calculated.

Senescence-associated β-galactosidase staining. After transfection with miR-34a mimics or inhibitors, PC12 cells were stained for SA-β-gal activity analysis. Cells were fixed with 4% formaldehyde for 15 min at room temperature, washed three times with PBS, and incubated with 1 ml X-gal solution (Hangzhou Sijiqing Biological Engineering Materials Co.) for 12 h at 37°C, avoiding exposure to CO₂. Following incubation, a blue color developed in senescent cells, observed under a microscope (IX71; Olympus Corporation, Tokyo, Japan) and the proportions of senescent cells were observed by digital imaging (Motic Images Plus 2.0; Motic China Group Co., Ltd., Xiamen, China).

Apoptosis analysis by fluorescent-activated cell sorting. After transfection with miR-34a mimics or inhibitors, PC12 cells were harvested, washed with ice-cold PBS, resuspended in 500 µl binding buffer, and incubated with 5 µl propidium iodide (PI; Beyotime Institute of Biotechnology, Jiangsu, China) and 5 µl Annexin V-fluorescein isothiocyanate (FITC; Beyotime Institute of Biotechnology) for 10 min in the dark. The cells were then washed and resuspended in 500 µl PBS, and cell apoptosis was analyzed by flow cytometry (using an Epics XL-MCL flow cytometer; Beckman Coulter, Inc., Brea, CA, USA).

Western blot analysis. PC12 cell lysates were extracted using a BCA Protein Assay Reagent kit (Beyotime Institute of Biotechnology, Shanghai, China), following mimic or inhibitor treatment. Protein concentration was determined using the bicinchoninic acid protein assay reagent (Beyotime Institute of Biotechnology). Equal quantities of protein from each sample were loaded and electrophoresed on 10% SDS-PAGE gels (Beyotime Institute of Biotechnology), and transferred to nitrocellulose membranes [Sangon Biotech (Shanghai) Co., Ltd., Shanghai, China]. After blocking with non-fat dried milk, membranes were probed with polyclonal rabbit anti-human acetyl-p53 [dilution, 1:1,000 (cat. no. CS2525); Cell Signaling Technology, Inc., Danvers, MA, USA], polyclonal rabbit anti-human Bcl-2 [dilution, 1:1,000 (cat. no. BS4023); Bioworld Technology, Inc., St. Louis Park, MN, USA] or polyclonal rabbit anti-human SIRT1 [dilution, 1:1,000 (cat. no. CS2327); Cell Signaling Technology, Inc.] antibodies. Antibody signals were visualized using a Chemiluminescent Detection kit, according to the manufacturer's instructions (Beyotime Institute of Biotechnology). Experiments with blank and negative controls were conducted in parallel. The relative band intensities of the blots were quantified with Adobe Photoshop software (Adobe Systems, Inc., San Jose, CA, USA).

**Statistical analysis.** All experiments were repeated at least three times. All values are expressed as the mean ± standard deviation. The difference between means was analyzed by Student's unpaired t-test. P<0.05 was considered to indicate a statistically significant difference. All statistical analyses were conducted using SPSS 19.0 (SPSS, Inc., Armonk, NY, USA).

**Results**

miR-34a mimics inhibit the proliferation of PC12 cells. To assess the biological role of miR-34a during the proliferation of PC12 cells, cells were transiently transfected with miR-34a mimics or inhibitors, and the proliferation was measured 24, 48 and 72 h following transfection using an MTT assay. As shown in Fig. 1, following transfection with miR-34a mimics, the proliferation of PC12 cells was significantly decreased compared with that of the negative control group (P<0.01). The proliferation of the miR-34a inhibitor group was marginally...
higher than that of the negative control group; however, no significant difference was identified (P>0.05).

**miR-34a induces PC12 cell apoptosis.** It is well known that miR-34a is an important component of the p53 tumor suppressor protein transcriptional network, which regulates cell proliferation and cell cycle progression. In order to investigate the biological effects of miR-34a in nerve cells, PC12 cells were transiently transfected with miR-34a mimics or miR-34a inhibitors, and the proportions of apoptotic cells were quantified using...
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An Annexin V-FITC/PI dual staining assay. As shown in Fig. 2, after transfection, the total proportion of apoptotic cells in the miR-34a mimic group was significantly increased compared with that of the control group (P<0.01). The apoptotic rate of miR-34a inhibitor group was marginally lower than that of the control group, however, no significant difference was identified (P>0.05). The total apoptotic rates for control, miR-34a inhibitor and miR-34a mimic transfection groups were 12.2±1.06, 11.4±0.34 and 19.2±0.84%, respectively.

miR-34a induces PC12 cell senescence. The influence of miR-34a on cell senescence was then evaluated using the β-galactosidase staining assay. The number of non-adherent cells in the miR-34a mimic group was increasing compared with the miR-34a inhibitor group and the control group, however, no significant difference was identified between the miR-34a inhibitor group and the control group (Fig. 3A-C). SA-β-gal staining analysis showed that the miR-34a mimics greatly increased SA-β-gal activity (Fig. 3D-F). These results demonstrate that miR-34a increases PC12 cell apoptosis and senescence.

miR-34a reduces the expression of Bcl-2 and SIRT1. To determine whether miR-34a expression levels correlate with the ac-p53, Bcl-2 and SIRT1 levels in PC12 cells, the expression of each protein was quantified by western blot analysis after transfection with miR-34a mimics or inhibitors. As Fig. 4 shows, compared with the control group, the expression levels of Bcl-2 (P<0.01) and SIRT1 (P<0.05) in the miR-34a mimic group were significantly reduced, while the levels of ac-p53 (P<0.05) were elevated in this group. In addition, the ac-p53 in miR-34a inhibitor group was reduced compared with the control group (P<0.05). The levels of SIRT1 in the miR-34a inhibitor group were significantly elevated compared with the miR-34a mimics group (P<0.05), however, the SIRT1 levels were not significantly different from the control group. The levels of Bcl-2 in the miR-34a inhibitor group were marginally elevated compared with the miR-34a mimic and the control groups, however the difference was not significant.

Discussion

PC12 is a cell line derived from a pheochromocytoma of the rat adrenal medulla, which contains a mixture of neuroblastic and eosinophilic cells (16). PC12 cells stop dividing and terminally differentiate when treated with nerve growth factor or dexamethasone, rendering PC12 cells a good in vitro model for investigating neuronal differentiation and neurosecretion (17,18). Additionally, due to their widespread availability and transfectable features, PC12 cells are one of the most commonly used models for investigating the physiology, pathology and pharmacology of neural cell differentiation. In recent years, researchers have developed various neurodegenerative disease cell models, including Alzheimer's and Parkinson's disease models, using PC12 cells (19,20). As small, endogenously expressed non-coding RNAs, miRNAs regulate gene expression by promoting the degradation of target mRNA and inhibiting translation. miRNAs are frequently involved in the regulation of cellular differentiation, proliferation, metabolism and apoptosis. As a member of the miR-34 family, miR-34a has been widely investigated in recent years. Several studies have indicated that upregulation of miR-34a expression can induce apoptosis, senescence, differentiation, cell cycle arrest and growth suppression (21,22). Overexpression of miR-34a increases the proportion of postmitotic neurons of mouse neural stem cells (23). SIRT1 is a nicotinamide...
adenine dinucleotide (NAD)-dependent histone deacetylase that has been implicated in inflammation, circadian rhythms, hypoxic responses, cell survival, life longevity and metabolic processes (24,25). SIRT1 also exhibits a protective role in certain neurodegenerative disease models (26). It has been reported that SIRT1 inhibits lipopolysaccharide-mediated proinflammatory cytokine release in microglia and circumvents dopaminergic neuronal injury induced by activated microglial-derived factors via p53-caspase-3-dependent apoptosis, which indicates that upregulation of SIRT1 may provide a promising target for therapeutic intervention in neuroinflammatory diseases (27).

p53 is a sensor of chronic and acute alterations in cellular physiology and interacts with DNA to aid in regulating chromosomal integrity (28). miR-34a enhances p53 activity by reducing p53 deacetylation, which in turn results in a decrease in SIRT1 expression (15). This decrease is achieved at the post-transcriptional level, involving miRNA binding to the 3'UTR of SIRT1. In addition, the inhibition of SIRT1 activates p53-dependent apoptosis through deacetylation and stabilization of p53. As an important anti-apoptosis gene, Bcl-2 cooperates with apoptosis activating factors and forms the Bcl-2-Apaf-1-caspase 9 complex, which inhibits the activation of caspases 9 and 3, and prevents the initiation of mitochondrial apoptosis access. A study showed that miR-34a inhibits the function and activity of Bcl-2 and promotes cellular apoptosis (29).

As demonstrated in the present study, transfection of PC12 cells with miR-34a mimics resulted in insignificant alterations in cell viability compared with the control group. Apoptosis analysis by flow cytometry revealed that the apoptosis rate of the miR-34a mimic group was significantly higher compared with the control group. Consistently, with the control group, the expression of Bcl-2 and SIRT1 in the miR-34a mimic group was decreased, while the ac-p53 was increased. Cell senescence is a form of stagnation of cell growth and, the results obtained using the SA-β-gal staining assay showed that the miR-34a mimic greatly increased the SA-β-gal activity, suggesting that the miR34a mimic could increase the senescence of PC12 cells. miR-34a binds to the 3'-UTR sequences of SIRT1 mRNA, immediately inhibiting the translation of SIRT1. The decrease in SIRT1 expression leads to an increase in the acetylation of p53, enhancing p53 transcriptional activity, through the positive feedback loop as shown in Fig. 5. As a highly-conserved NAD+ induced deacetylase enzyme, SIRT1 was first identified in yeast, where, through regulation of downstream targets, such as p53, Foxo and Ku79, it is involved in reducing oxidative stress and apoptosis, and regulating gene silencing and cell cycle progression (30-32). SIRT1 promotes the deacetylation of ε-terminal lysine 382 of the p53 protein, regulating the transcriptional activity of p53 and inhibiting p53-induced apoptosis (33). It is also reported that p53 can inhibit autophagy by reducing expression of SestrinZ and Draln, and accelerating cellular senescence (34,35). Studies have revealed that the deposition of β-amyloid protein (Aβ) is a critical initiating factor in Alzheimer's disease, and Aβ is a direct contributor to the neurofibrillary tangles in the brain and results in the loss of neurons (36). In models of Alzheimer's disease, high expression of SIRT1 in rat brains was found to strengthen the catabolic pathway of the amyloid protein, and reduce Aβ deposition (37). The present study showed that transfection of miR-34a mimics can effectively reduce the expression of SIRT1 in PC12 cells, while transfection of miR-34a inhibitors can increase the expression of SIRT1. Therefore, it was demonstrated that miR-34a activates SIRT1, which, due to the roles of SIRT1 in Alzheimer's disease, indicates that miR-34a may aid in preventing and curing Alzheimer's disease.

In conclusion, the present data indicates that miR-34a induces PC12 cellular apoptosis, which may be associated with the inhibition of SIRT1 and Bcl-2. Furthermore, this study highlights the importance of the positive feedback loop formed by miR-34a-SIRT1-p53 in cellular apoptosis. These results reveal that miR-34a is a key regulator of cellular apoptosis and a potential therapeutic target in neuronal diseases.

Acknowledgements

This study has been supported by the National Science Foundation of China (grant no. 81372108).

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