Original Paper

The Effects of miR-136-5p-Mediated Regulation of A20 in Astrocytes from Cultured Spinal Cord Cultured Cells *In Vitro*

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Key Words

miR-136-5p • Astrocyte • IL-17 • A20

Abstract

Background/Aims: This study focused on investigating the regulatory mechanism of miR-136-5p in mouse astrocytes stimulated with interleukin-17(IL-17). **Methods:** C57BL/6 mouse astrocytes were stimulated with IL-17 (100ng/ml) for various periods of time (0-48 hours) and at various doses (0-200 ng), and the expression levels of inflammatory cytokine and chemokine genes (IL-6, TNF- α , MCP-1, MCP-5 and MIP-2) were then detected by real-time PCR. The expression of the A20 gene was measured with real-time PCR in cells that were stimulated with IL-17 (50 ng/ml) for various periods of time (0-48 hours). C57BL/6 mouse astrocytes were transfected with Ctrl-anti-miR-136-5p or LNA -anti-miR-136-5p for 48 h. Thereafter, the cells were stimulated with or without IL-17 (50ng/ml) for 6 h. The level of A20 protein (TNF α -induced protein 3, TNFAIP3) was detected by Western blot analysis. **Results:** (1) Compared with the DMEM control group, within six hours, IL-17 stimulation significantly increased the expression levels of inflammatory cytokine and chemokine genes and clearly decreased the expression level of the A20 protein. (2) Without IL-17 stimulation, the expression level of the miR-136-5p gene was significantly decreased, whereas in the miR-136-5p-inhibition group, the A20 protein expression was elevated. IL-17 stimulation slightly decreased the expression of the A20 protein in the miR-136-5p-inhibition group, but it was still slightly higher than in the control group. **Conclusion:** This study demonstrated that miR-136-5p affected the expression of A20 in IL-17-stimulated astrocytes. .

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Introduction

Astrocytes are the most abundant cell type in the mammalian central nervous system (CNS), and they constitute approximately 90% of human brain tissue. Astrocytes have been

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considered both an ally and an enemy in the fight against CNS immune infiltration and the fight for the restoration of neuronal function. Astrocytes play important physiological signaling and immunological roles in CNS homeostasis [1-4]. During CNS inflammation, astrocytes can release abundant proinflammatory cytokine and chemokine that then activate autoreactive T cells. Subsequently, the T cells produce chemokines and inflammatory cytokines that promote secondary waves of immune cell infiltration into the CNS [5-7]. Moreover, the main cause of the multiple sclerosis (MS) plaques is the proliferation of astrocytes, which increase the expression of glial fibrillary acidic protein (GFAP) upon activation by astrogliosis [8]. Given that astrocytes mitigate inflammation in CNS injury, they could represent a highly effective target for MS treatment if interference with their protective properties can be avoided [9-11].

Th17 cells play critical roles in the effector mechanisms of autoimmune diseases. IL-17 is a family of proinflammatory cytokines that consists of six ligands (IL-17A, B, C, D, E and F) that are produced by a subset of Th17 cells and that bind to their associated heteromeric transmembrane receptors (IL-17RA, B, C, D and E). Because they lack IL-17 and the IL-17 receptor, mice are less susceptible to the experimental induction of autoimmune encephalomyelitis (EAE) induction [12-14]. IL-17-specific inhibition attenuates inflammation, which suggests that IL-17 signaling plays an important role in the effector stage of EAE [15]. Astrocytes constitutively express the IL-17 receptor, which allows IL-17 to exert direct effects on astrocytes, particularly in a variety of autoimmune diseases, such as multiple sclerosis, asthma, arthritis inflammatory bowel disease, psoriasis and rheumatoid arthritis. The expression level of the IL-17 protein is significantly up-regulated. Astrocytes subjected to IL-17 stimulation produce abundant inflammatory cytokines and chemokines and concomitantly initiate astrogliosis, which aggravates EAE lesions [16, 17]. A recent study found astrogliosis in IL-17A/GFAP transgenic mice following histological characterization. However, the detailed mechanism responsible for IL-17-mediated astrogliosis is not clear [18]. Many studies have indicated that microRNAs (miRNAs), which are a class of non-coding RNAs that modulate gene expression via the degradation of mRNAs or the inhibition of translation, play important roles in the development of the spinal cord and the pathological changes that occur following spinal cord injury, and thus some miRNAs may be effective targets for therapeutic interventions following spinal cord injury [19]. A20 is recognized as a central regulator of inflammation and is a widely inducible cytoplasmic protein that is associated with several autoimmune disorders, including MS. However, the miRNA-related mechanisms that operate both in the brain tissues of EAE mice and in astrocytes that are stimulated with IL-17 remain unknown [20].

In this study, we observed changes in the miRNA profiles of astrocytes that were stimulated with IL-17 *in vitro*. We selected miR-136-5p, which is a co-up-regulated miRNA. Our work revealed that miR-136-5p facilitated the production of inflammatory cytokines and chemokines in astrocytes that were stimulated with IL-17 *in vitro*. Our findings suggest that targeting certain miRNAs in astrocytes might represent an important option for combating MS and EAE. This study also describes the role and mechanism of miR-136-5p in the development of central nervous system injury.

Materials and Methods

Ethics statement

This project fully considered and protected the rights and interests of the study subjects. This study also met the criteria of the Ethical Review Committee. The Medical Ethics Committee of the First Affiliated Hospital of Guangxi Medical University approved the protocol.

Materials

Fetal bovine serum (FBS) and DMEM/F12 medium were purchased from HyClone (Logan, UT, USA). Recombinant murine IL-17 was purchased from Peprotech (Rocky Hill, New Jersey, USA). Trypsin was

purchased from Sigma-Aldrich (St. Louis, MO, USA). A20 antibody and β -actin rabbit polyclonal antibody were purchased from Abcam (Cambridge, Massachusetts, USA). Regarding the experimental animals and cells, the astrocytes were isolated from the cerebral cortices of 0-2-days-old C57BL/6 mice (Experimental Animal Center of Guangxi Medical University).

Methods

Isolation, primary culture and subculture of the mouse astrocytes in vitro. Based on the reference literature [14, 21, 22], 0-2-days-old C57BL/6 mice were used. After sacrifice, the mice were soaked in 75% ethanol for 3-5 min, and the heads were cut off under sterile conditions with ophthalmic scissors. The skulls were carefully cut open, and both cerebral hemispheres were then removed. Under a microscope, the meninges were peeled away, and the cerebral hemispheres were placed in a sterile petri containing DMEM/F12 culture medium. Then, DMEM/F12 culture medium was used to rinse the hemispheres twice, and the cerebral cortex was minced. The tissue was digested in a 0.25% trypsin solution at 37°C for 10 min, and digestion was terminated by using complete DMEM/F12 medium with 10% fetal bovine serum and gently pipetting. The resulting solution was centrifuged at 800 RPM for 5 min. Ten milliliters of DMEM/F12 medium was added, and a pipette was used to mix the solution. The solution was centrifuged and washed twice. The cells were placed in a culture incubator at 37°C with 5% CO₂. The cells were cultured for 24 h, and the medium was changed. The culture medium was replaced every 2-3 days, and cell growth was observed under an inverted microscope. Cells were grown to 80-90% confluence, and then, the cells were digested and subcultured at a ratio of 1:3.

Identification of astrocytes. Astrocytes have special morphology that is established by glial fibrillary acidic protein (GFAP). Purified astrocytes were stained with an anti-GFAP antibody using immunohistochemical staining (SABC method) or immunofluorescence staining. Astrocytes in the third passage were established at a density of 1.0×10^6 /ml in a 12-well culture plate; the inside of each well contained a coverslip. The coverslip was covered with PLD. The cells were cultured for 3-5 days in an incubator at 37°C and with 5% CO₂, and then, GFAP staining was used to identify astrocytes.

Construction of the miR-136-5p lentiviral vector and determination of viral titer. The design of miR-136-5p-inhibitor and miR-136-5p-reverse were determined by the Genechem Biological Company. The lentiviral vector synthesis used a three-plasmid expression system that included the GV280 plasmid, the pHelper 1.0 vector and the pHelper 2.0 vector. The miR-136-5p-inhibition and the control recombinant lentiviral expression vectors were synthesized by the Genechem Biological Company and subsequently verified by sequencing. Finally, the lentiviral titer was determined.

Infection of astrocytes with LV-miR-136-5p-inhibition lentivirus. (1) Third passage astrocytes were inoculated in 6-well plates. When the cell density reached approximately 80%, the virus was added.

(2) Thirty microliters of lentiviral supernatant were added to 1 ml of 10% FBS and antibiotic-free DMEM/F12 culture medium, and polybrene was added (a final concentration of 5 μ g/ml).

(3) The cell culture fluid was removed, and the virus diluent from step (2) was added to each well. To establish the controls (the blank and negative controls), the culture conditions were 37°C and 5% CO₂ for 12-24 h.

(4) The virus suspension was removed, and 1 ml of antibiotic-free DMEM/F12 medium containing 10% FBS was added. The culture was maintained at 37°C and 5% CO₂ for 48-72 h.

(5) Fluorescence microscopy was used to observe the infection rate of the virus.

Experimental Group

To study the effect of IL-17 on the expression of miR-136-5p, the levels of A20 mRNA and protein in the mouse astrocytes were probed. According to whether the cells were transfected with the virus, the samples were divided into 6 groups: the DMEM blank group, control groups, and the miR-136-5p-inhibition groups with and without IL-17. After 6h, the cells from each group were collected. RT-PCR was used to detect levels of the miR-136-5p and A20 mRNAs in the mouse astrocytes. Western blotting was used to detect the expression level of the A20 protein.

Real-time PCR to detect the expression of miR-136-5p in mouse astrocytes

The total RNA separation and extraction methods were performed according to the instructions of the TRIzol kit, and a UV fluorescence spectrometer (ND-1000) was then used to determine the ratios

Table 1. Sequences of the primers used for PCR

Molecule	Sequences (5'-3')
mmu-miR-136-5p-inhibition	Forward 5'-AATTCAAAAACTCCATTGTTTGTGATGATGG-3' Reverse 5'-CgggCCATCATCAAAACAAATGGAGTTTTTg-3'
IL-6	Forward 5'-CCACGGCCTTCCCTAC-3' Reverse 5'-AAGTGCATCGTTGT-3'
TNF- α	Forward 5'-CATCTTCTCAAAATTCGAGTGACAA-3' Reverse 5'-TGGGAGTAGACAAGGTACAACCC-3'
MCP-1	Forward 5'-CAGCCAGATGCAGTTAACGC-3' Reverse 5'-GCCTACTCATTGGGATCATCTTG-3'
MCP-5	Forward 5'-GGAAGCTGAAGAGCTACAGGAGAA-3' Reverse 5'-GAAGGTTCAAGGATGAAGGTTTGA-3'
MCP-2	Forward 5'-GCCCCTCCACCTGCCGGCTGC-3' Reverse 5'-CTGAACCAGGGGGCTTCAGGG-3'
A20	Forward 5'-TGGGAGCAGTGTTAAAGGCA-3' Reverse 5'-TCGGAAGTGTGGGCAAACT-3'
β -actin	Forward 5'-ATGGCTGGGGTGTGAAGGT-3' Reverse 5'-ATCTGGCACCACACCTTCTACAA-3'

of absorbance at 260/280 and A260/230 to determine the purities and concentrations of the total RNA samples. Reverse transcription reactions were performed using 500 ng of RNA. U6 was used as a reference. Table 1 lists the primer sequences. The qPCR reaction conditions were as follows: s: 42°C for 60 min and 70°C for 5 min; using, 2x SYBR Green qPCR mix 10 μ l, 10 μ mol/L forward primer 1 μ l and 10 μ mol/L reverse primer 1 μ l. Water was added to achieve a total volume of 20 μ l, and each sample containing miRNA and U6 was examined using an ABI 7500. The experiment was repeated 3 times, and the data were analyzed using the $2^{-\Delta\Delta C_t}$ method.

Western blotting to detect the A20 protein expression level in the mouse astrocytes

After each group of cells was cultured in 6-well plates for 6 hours, 1 ml of protein lysate containing 0.1 mmol/L PMSF was added, and the cells were left to lyse for 30 minutes at 4°C. Each cell lysate was collected at 4°C and then centrifuged for 10 minutes at 13,400 g. The supernatants were collected, and the BCA method was used to determine the protein contents. The proteins were subjected to electrophoresis (SDS-PAGE) and transferred to PVDF membranes in semi-dry conditions. Five percent milk was added to the PVDF membrane, which was then incubated at 37°C for 1 h. Then, the anti-A20 (1:500) antibody was applied. The membranes were left at 4°C overnight. The secondary antibody was applied (1:2,000), and the samples were left to incubate at 37°C for 1 h. Color imaging was performed using Image-Pro Plus software.

Statistical analysis

All of the experimental data were analyzed using the SPSS 19.0 statistical software. The data are presented as the mean and standard deviation (mean+ S.D.) of each group, and $P < 0.05$ was considered statistically significant. ANOVAs were performed to compare the data between groups, and LSD -t-tests were also performed to compare the groups.

Results

Primary culture and identification of the mouse astrocytes

Under an inverted microscope, the astrocytes were irregular in shape. The astrocytes had abundant cytoplasm with relatively minimal, nucleoplasm in the multi- branching soma. The nuclei were oval and often located on one side of the cell. The rabbit anti-GFAP polyclonal antibody was used to immunocytochemical and immunofluorescence staining. After staining, GFAP was found in the cytoplasm. To determine the percentage of positive cells ((number of positive cells/ total cell number) $\times$ 100 in 5 fields), the number of positive cells and the total number of cells were counted, and the percentage of of GFAP positive staining was calculated to be greater than 95%.

Fig. 1. Optimization of the stimulation time and concentration for mouse astrocytes. a: Cells were stimulated with IL-17 (100 ng/ml) for different lengths of time (3, 6, 12, 24 and 48 hours). The relative mRNA levels of IL-6, TNF- α , MCP-1, MCP-5 and MIP-2 were detected by real-time PCR. The results are expressed as the means $\pm$ SD. * $P < 0.05$ and ** $P < 0.01$ compared with the DMEM control group. # $P < 0.05$; ## $P < 0.01$ compared with the IL-17 (6 h) -stimulated group. b: The cells were stimulated with different concentrations of IL-17 (10, 20, 50, 100, and 200 ng/ml) for 6 h. The relative mRNA levels of IL-6, TNF- α , MCP-1, MCP-5 and MIP-2 were detected by real-time PCR, respectively. * $P < 0.05$ and ** $P < 0.01$ compared with the control group; # $P < 0.05$ and ## $P < 0.01$ compared with the IL-17 (50 ng/ml)-stimulated group.

Optimization of the stimulation time and the concentration of the mouse astrocytes

To determine the optimal duration of exposure to IL-17, we used IL-17 (100 ng/ml) to stimulate the cells for 3, 6, 12, 24 and 48 hours. Next, real-time PCR was used to measure the mRNA abundance of IL-6, MIP-2, MCP-1, MCP-5 and TNF- α (Fig. 1a). The mRNA levels of IL-6, MIP-2, MCP-1, MCP-5 and TNF- α began to rise in the third hour. After the sixth hour, the levels significantly increased and reached their peaks. After the twelfth hour, there was a slight decline. At the twenty-fourth hour, the levels were still significantly higher than those of the control group. Therefore, we selected 6 hours as the optimal stimulation time for the mouse astrocytes.

To determine the optimal concentration of IL-17, we stimulated the cells for 6 hours using different concentrations of IL-17 (10 ng/ml, 20 ng/ml, 50 ng/ml, 100 ng/ml, and 200 ng/ml). Next, real-time PCR was used to detect the mRNA abundances of IL-6, TNF- α , MCP-1, MCP-5 and MIP-2 (Fig. 1b), at the concentration of 10 ng/ml, the abundances of IL-6, TNF- α , MCP-1, MCP-5 and MIP-2 mRNA began to rise. When the dose was increased to 100 ng/ml, the mRNA expression levels exhibited slight declines. Therefore, the IL-17 concentration of 50 ng/ml was chosen as the condition for subsequent experiments.

Effect of IL-17 on the expression of A20 in mouse astrocytes

To determine the optimal duration of exposure to IL-17, we used IL-17 (50 ng/ml) to stimulate the cells for 3, 6, 12, 24 and 48 hours. Next, real-time PCR was used to measure

Fig. 2. The relative mRNA expression levels of A20 with IL-17-stimulation. The Mouse astrocyte cells were stimulated with IL-17 (50ng/ml), and the relative expression levels of A20 were detected by RT-PCR at different times (3, 6, 12, 24 and 48 hours). The results are expressed as the means $\pm$ SD. *P<0.05 and **P<0.01 compared with the group-stimulated with IL-17 (50ng/ml) for 6 h.

Fig. 3. The relative mRNA expression levels of miR-136-5p. Mouse astrocytes were transfected with control or anti-miR-136-5p virus for 48 h. The mRNA Expression of miR-136-5p was detected by real-time PCR. The results are expressed as the mean $\pm$ SD. *P<0.05, **P <0.01 Compared with the blank group; #P<0.05; ## P<0.01 Compared with blank group stimulated with IL-17 for 6h.

Fig. 4. Western blot analysis of A20. After IL-17-stimulation (50ng/ml) for 6 hours, the expression of the A20 protein was detected by Western blotting. The results are expressed as the mean $\pm$ SD. *P<0.05; **P<0.01 compared with the control group; #P<0.05; ##P <0.01 compared with the control group stimulated with IL-17 for 6h.

the mRNA abundance of A20. As illustrated in Figure 2, the abundance of A20 mRNA began to decrease in the third hour. After the sixth hour, the levels significantly decreased and reached a minimum. After the twelfth and twenty-fourth hours, there were slight increases. Therefore, we selected 6 hours as the optimal stimulation time for mouse astrocytes.

Effect of IL-17 on the expression of miR-136-5p in the mouse astrocytes

Analysis of the expression level of miR-136-5p in the mouse astrocytes following transfection with the slow lentivirus suspension revealed that the slow virus suspension (the miR-136-5p-inhibition virus or the negative control virus) infected the mouse astrocytes. RT-PCR was used to detect the miR-136-5p expression level in mouse astrocytes. The results revealed that there was no significant difference in the levels of miR-136-5p between the negative control group and the blank group. Analysis of miR-136-5p expression levels in the mouse astrocytes after stimulation with IL-17 revealed that, compared with negative control group, the content of miR-136-5p in the miR-136-5p-inhibition group was significantly reduced. The relative expression levels of miR-136-5p were significantly

increased in the negative and control groups, but there was no significant difference in the relative expression of miR-136-5p between the latter two groups (Fig. 3).

The expression of A20 protein in mouse astrocytes following IL-17 stimulation as assessed with Western blotting

Without IL-17 stimulation, the expression of the A20 protein was significantly higher in the miR-136-5p-inhibition group than in the blank and control groups. There was no significant difference in the expression of the A20 protein between the blank group and control groups ($P > 0.05$). IL-17 stimulation slightly decreased the A20 protein concentration in the miR-136-5p-inhibitor group compared with the blank group and control groups. However, the expression of A20 protein was still higher in the miR-136-5p-inhibition group. There was no significant difference in the expression of the A20 protein between the blank and group and control groups (Fig. 4).

Discussion

MicroRNAs (miRNAs) are a class of endogenous non-coding RNAs that serve regulatory functions, and they are approximately 20-25 nucleotides in length. Mature miRNAs originated from long primary transcripts that are cleaved by nucleic acid enzymes. Recent studies suggest that miRNAs are involved in a wide variety of regulatory pathways, including development, virus defense, hematopoiesis, organ formation, cell proliferation, apoptosis and fat metabolism. [21-23]. A variety of miRNAs are involved in the development of the nervous system and in pathological processes of the nervous system as well. The effects of mmu-miR-136-5p transcriptome on blastocysts were measured with an miRNA TaqMan assay-based method and qRT-PCR. This analysis further revealed that the increased expression of miR-136-5p may regulate the implantation of embryos [24]. Skeletal muscle development is a complex biological process that is regulated by numerous genes and non-coding RNAs, such as microRNAs. A previous study indicated that miR-136-5p plays an important role in skeletal cytotogenesis in goats [25]. Another study found that there is a direct effect of ultraviolet radiation (UVR) on the modulation of miR-136-5p that affects the translation and stability of mRNAs in the dorsal skin of SKH1 mice (6-7 week old 7-weeks old) that are exposed to acute and chronic doses of ultraviolet ultraviolet radiation [26]. The serum profiles of miR-136-5p are up-regulated in patients with TSC, and after three months of mTOR inhibitor treatment, significant increases in miR-136-5p over baseline levels [27]. MiR-136-5p is enriched in neurons preclinical disease, and it likely regulates local protein synthesis during rapid responses to stressors, such as replicating prions [28]. These findings imply that miR-136-5p may play an important role in neuronal disease.

A20 is a classical negative immune regulatory protein. A20 is involved in the regulation of the activation of transcription factor NF- κ B via E3 ubiquitin ligases. A20 protein-deficient mice spontaneously exhibit severe inflammatory diseases and die during the perinatal period [29-31]. Single nucleotide polymorphism analysis revealed that the A20 gene is linked to some human autoimmune diseases, such as systemic lupus erythematosus, rheumatoid arthritis, EAE and AS, which implies that A20 is involved in human autoimmune homeostatic regulation [32, 33]. A20 is not only involved in the occurrence and development of various autoimmune and inflammatory diseases; it also plays important roles in tumor- and virus-induced immune tolerance [34]. Therefore, changes in the expression and activity of A20 will affect the intensity and duration of the immune response [35-37]. The regulation of the expression of A20 has the ability to change the body's immune state to adapt to different immune responses, which highlights the value of A20 as an immune target molecule.

In our study, we transfected mouse astrocytes with a miR-136-5p-inhibition viral vector to reduce the miR-136-5p content in the cell and detected the expression of miR-136-5p that was induced by IL-17 in astrocytes. We further investigated whether miR-136-5p could regulate A20 protein expression in mouse astrocytes that had been stimulated with the IL-

17 protein. The results revealed that without IL-17 stimulation, the expression of the A20 protein was significantly lower in the the miR-136-5P-inhibition group than in the blank group. Without the IL-17 stimulation, the expression of miR-136-5P was significantly decreased, and the protein expression of A20 was significantly increased in the miR-136-5P-inhibition group. IL-17 stimulation slightly decreased A20 protein expression in the miR-136-5p-inhibitor group, compared with the blank and negative groups. However, the expression of the A20 protein was higher in the miR-136-5p-inhibition group than in the blank and negative groups. Based on these experimental results, we believe that miR-136-5p regulates the expression of the A20 protein in IL-17-stimulated astrocytes. IL-17 induces aberrant miRNA expression in brain astrocytes. The co-expressed miRNA (miR-136-5p) was able to regulate the production of inflammatory cytokines and chemokines *in vitro*, which would result in effects on the development of EAE. Thus, these findings provide hints regarding the roles of miRNAs and suggest a novel therapeutic target for this autoimmune disease.

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Disclosure Statement

The authors declare that there are no conflicts of interest.

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