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Short communication

Effects of *Echinococcus multilocularis* miR-71 mimics on murine macrophage RAW264.7 cells

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ABSTRACT

The microRNAs (miRNAs) are a class of small regulatory non-coding RNA that contributes to the activation of host-pathogen cross-talk during infection. In helminthes, miR-71 is highly conserved and it has recently been detected in nematode exosomes, as well as in the sera and/or fluids of infected humans and mice. However, the role of miR-71 during infection remains poorly characterized. Herein, we show that *Ago1* and *Ago4*, which encode key components of the small RNA-induced silencing complex (RISC), were up-regulated in murine macrophage RAW264.7 cells transfected by *Echinococcus multilocularis* miR-71 (emu-miR-71) mimics. Using a miRNA PCR array, none of the 84 miRNAs involved in inflammation or autoimmunity were significantly up- or down-regulated in the transfected cells ($p > 0.05$). Although it did not influence IL-10 production by the treated cells ($p > 0.05$), the mimics significantly repressed the production of NO 12 h after treatment with LPS and IFN-γ ($p < 0.01$), identifying another potential mechanism whereby parasites can carefully regulate host levels of NO. These findings indicate that the release of parasite-derived miR-71 into hosts can affect the functions of macrophages, and possibly represents an exciting direction for studies of the interplay between parasites and hosts.

1. Introduction

During infection, *Echinococcus* species have evolved multiple ways to reside peacefully within their respective host without triggering damaging immune responses, including the release of reactive inflammatory substances [1]. The microRNAs (miRNAs) are a class of small regulatory non-coding RNAs that are ~21 bp and can bind to the 3′-UTR of mRNAs to inhibit translation or promote decay. miRNAs have been recently shown to act as molecules that allow modulation of host innate immunity via the secretion of miRNA-containing exosomes, a type of multivesicular body-origin extracellular vesicles with a diameter of 50–250 nm [2], and exosome release has been found to occur during nematode infection [3]. Parasite-derived miRNAs have been shown to be present in serum and/or fluids of humans and animals that are infected with nematodes [3–5] or trematodes [6]. Such miRNAs in the serum have been proposed to be associated with the intensity of infection [6], but the roles of these molecules in vivo remain largely unknown.

In helminthes, miR-71 is ubiquitously present and conserved, and is genetically clustered with other miRNAs, such as miR-2a/-2b/-2c, in platyhelminths [3,7]. Furthermore, miR-71 is involved in longevity [8,9] and neuron development [10] in nematodes, and is potentially involved in the biological functions of neoblasts, which are the only known type of cell with the ability to undergo mitosis in platyhelminths [11]. It has been shown that the presence of parasite-derived miR-71 can be detected in the serum, plasma, and/or lymph of hosts, including humans infected by nematodes [3–5] or trematodes [6], suggesting that a conserved miR-71-secretion system might exist in helminthes. Moreover, nematode miR-71 can also be released into the host via exosomes that can be internalized by host cells and act as regulator of innate immunity [3]. Although the role of miR-71 secreted by parasites remains unclear, it has been proposed that it plays a role in host-pathogen interactions [3,5]. Using *Echinococcus multilocularis* miR-71 (emu-miR-71) mimics, we show that the mimics induced increased expression levels

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of Argonaute (Ago) 1 and 4, and inhibited NO production early after LPS + IFN-γ stimulation, but did not exhibit effects on cytokine production or the expression of miRNAs known to be related to inflammation or autoimmunity.

2. Materials and methods

2.1. Cell culture and transfection

Murine macrophage RAW264.7 cells were maintained in RPMI-1640 (Invitrogen, California, USA) media supplemented with 10% fetal bovine serum (Invitrogen) and antibiotics (Sangon Biotech, Shanghai, China) in a 37 °C incubator with 5% CO_2. At 1 day prior to transfection or stimulation, cells at ~80% confluence were harvested using trypsin detachment solution (Sigma–Aldrich, St. Louis, MO, USA) and cell viability was confirmed based on trypan blue exclusion (Sigma–Aldrich). Unless otherwise specified, 5.0×10^4 and 2.0×10^5 cells were seeded in 96- and 24-well plates (Costar, Washington, USA), respectively. When cell confluence reached ~70–80%, cells were transfected by emu-miR-71 mimics (Invitrogen), an artificially-synthesized analogue to emu-miR-71, or negative control constructs (Invitrogen) using RNAiMAX (Invitrogen), respectively, according to the manufacturer's instructions. The media was replaced at 12 h post-transfection and then cells were further incubated for 12 h. Transfected cells were used for subsequent stimulation experiments.

2.2. RNA extraction

All reagents for RNA extraction were prepared using DEPC-treated water. Cells were washed three times with ice-cold PBS, followed by the addition of TRIzol to recover total RNA according to the manufacturer's instructions with some modifications (Invitrogen). In brief, after homogenization and centrifugation, supernatants were used for RNA precipitation by adding isopropanol and 1 μl 10 mg/ml glycogen (Invitrogen), and incubating overnight at −20 °C. Total RNA was resuspended in nuclease-free water. The concentration and integrity of extracted RNA samples were determined using a Nanodrop (Thermo Scientific) and formaldehyde-denaturing electrophoresis.

2.3. Measurement of NO produced by RAW264.7 cells

Generally, nitric oxide (NO) is unstable and can be easily oxidized into nitrite (NO_2^-) [12]. The levels of nitrite indirectly reflect NO output. After treatment with a final concentration of 100 ng/ml LPS and 10 ng/ml IFN-γ, levels of nitrite in the supernatant were determined using Griess reagent (Invitrogen). In brief, 100 μl culture supernatant was mixed with 13.33 μl Griess reagent and 86.67 μl nuclease-free water per well in a 96-well plate (Costar), followed by incubation in the dark at room temperature for 30 min. Absorbance values were read at 548 nm using a microplate reader (Bio-Rad, California, USA). Each sample was tested in triplicate and data used for the final analysis were pooled from three independent experiments.

2.4. Measurement of cytokine production by RAW264.7 cells

After stimulation with 100 ng/ml LPS, levels of IL-10 and IL-12 in the supernatant, two cytokines that play a crucial role in echinococcosis [1], were measured using DuoSet (R&D Systems) according to the manufacturer's instructions.

Briefly, 100 μl capture antibody diluted in filtered PBS was added in each well in 96-well plates (Costar), which were incubated overnight at room temperature and then washed three times with PBS containing 0.05% Tween-20. After washing, 300 μl Reagent Diluent was added into each well and then the plate was incubated at room temperature for 1 h. Next, 100 μl samples or standards were added per well and the plate was incubated at room temperature for 2 h, followed by the addition of 100 μl Detection Antibody per well and incubation at room temperature for 2 h. As a secondary antibody, 100 μl Streptavidin-HRP was added per well and the plate was incubated in the dark for 20 min at room temperature. Finally, 50 μl Stop Solution was added into each well and then was mixed thoroughly after the addition of Substrate Solution. Absorbance values were calculated by subtracting the readings at 540 or 570 nm from the readings taken at 450 nm. Each sample was tested in triplicate and data were pooled from three independent experiments.

2.5. Real-time PCR

The expression profiles of 84 miRNAs known to be involved in inflammation and autoimmunity were determined using a miScript miRNA PCR Array (Qiagen, Hilden, Germany). A mixture of 250 ng total RNA and 5× miScript Hispec Buffer were used as the initial input for the reverse transcription of miRNAs. Reactions were performed at 37 °C for 1 h, followed by inactivation and dilution. An overall 25 μl reaction volume that included diluted cDNA, 2× QuantiTect SYBR Green PCR Master Mix, and other molecules were added to each well. PCR was conducted using a Mx3005p thermocycler (Agilent, California, USA) according to the following steps: initial activation at 95 °C for 15 min, followed by 40 cycles of 94 °C for 15 s, 55 °C for 30 s, and 70 °C for 30 s. Data from triplicate independent experiments were evaluated using online software (http://pcrdataanalysis.sabiosciences.com/mirna).

Expression levels of 10 genes (*Ago1*, *Ago2*, *Ago3*, *Ago4*, *DgcR8*, *Dicer1*, *Drosha*, *Tarbp2*, *Xpo5*, and *Xrn2*) that encode key proteins involved in miRNA biogenesis were analyzed. First-strand cDNA synthesis was conducted using 2 μg total RNA with the ThermoScript™ RT-PCR System (Invitrogen). The reaction mixture of RNA, oligo $(dT)_{20}$, and other components was initially incubated at 65 °C for 5 min and then at 50 °C for 1 h. The reaction mixture was diluted by addition of 150 μl nuclease-free water. Real-time PCR was performed using All-in-One™ qPCR Mix (GeneCopoeia, Washington, USA) with the following thermocycling conditions: 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 s, 60 °C for 20 s, and 72 °C for 15 s. A total of four genes (*Actb*, *B2m*, *Sdha*, and *Gapdh*) were screened to identify an appropriate reference gene using online software (www.leonxie.com/referencegene.php). Validated primers for all of the above genes were purchased from GeneCopoeia. The relative expression levels of 10 genes were calculated using the $2^{-\Delta\Delta Ct}$ formula. Data used in the final analysis were collected from triplicate independent experiments.

2.6. Statistical analysis

GraphPad Prism 5 software was used for data analysis. Statistical significance was analyzed using a two-tailed unpaired *t*-test, unless otherwise indicated. *P*-values (<0.05) were considered to denote statistically significant differences.

3. Results and discussion

3.1. Effects of emu-miR-71 on key components of RISC

Compared to negative controls, we observed altered expression levels of *Ago1*, *Ago2*, *Ago3*, *Ago4*, *DgcR8*, *Dicer1*, *Drosha*, *Tarbp2*, *Xpo5*, and *Xrn2* after transfection of emu-miR-71 mimics, although only *Ago1* and *Ago 4* were markedly up-regulated by 1.44-fold and 1.69-fold, respectively (Fig. 1A). The mouse genome contains eight Argonaute-encoding genes, and *Ago1* and *Ago4* belong to the Ago subfamily that along with the Piwi subfamily constitutes the Argonaute protein family [13]. Both Ago1 and Ago4 can bind small RNAs, but lack catalytic activity [14], and they have both been shown to cooperate with Ago3 to silence multiple binding site-containing target mRNA transcripts [15]. Ago1 is closely associated with miRNA turnover as it