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Regular Article

Post-stroke treatment with miR-181 antagomir reduces injury and improves long-term behavioral recovery in mice after focal cerebral ischemia

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ABSTRACT

miR-181 has deleterious effects on stroke outcome, and reducing miR-181a levels prior to middle cerebral artery occlusion (MCAO) was shown previously to be protective. Here we tested the effect of post-ischemic treatment with miR-181a antagomir by intracerebroventricular and intravenous routes of administration on infarct size, neurological outcome, inflammatory response and long term behavioral outcome. Post-treatment with miR-181a antagomir significantly reduced infarction size, improved neurological deficits and reduced NF-κB activation, numbers of infiltrating leukocytes and levels of Iba1. Targets affected by miR-181a antagomir administered after stroke onset include BCL2 and X-linked inhibitor of apoptosis protein (XIAP). Post-treatment with miR-181a antagomir significantly improved behavioral outcome assessed by rotarod at one month. These findings indicate that post-treatment with miR-181a antagomir has neuroprotective effects against ischemic neuronal damage and neurological impairment in mice, and the protection is long lasting including recovery of motor function and coordination over one month. The ability to protect the brain with post-treatment with miR-181a antagomir with long lasting effect makes this a promising therapeutic target and may be an innovative and effective new approach for stroke therapy.

Introduction

Stroke is one of the leading causes of death worldwide and the leading cause of long-term neurological disability. Although many clinical stroke trials have been completed, the only clinically efficacious treatment to date is thrombolysis (Blakeley and Llinas, 2007). Suggested reasons for the many failures include the complex interplay among multiple signaling pathways and intracellular organelles, interaction between different cell types, and the potentially short therapeutic window for neuroprotection after stroke.

MicroRNAs (miRs) are a class of small, non-coding RNAs. Mature miRs are generated from primary miR transcripts by sequential endonucleolytic processing and act as posttranscriptional regulators of gene expression including in the setting of cerebral ischemia (for review see Ouyang et al. (2013)). Many miRs exist in families. The miR-181 family contains four highly conserved members, miR-181a, miR-181b, miR-181c and miR-181d, which are derived independently from 6 precursors located on 3 chromosomes as identified using TargetScan (http://targetscan.org).

Our previous study reported increased injury with increased levels of miR-181 and a protective effect of reducing miR-181a levels using antagomir when administered the day prior to middle cerebral artery occlusion (MCAO) in male mice (Ouyang et al., 2012b). Reduced levels of miR-181 were associated with reduced oxidative stress in *in vitro* ischemia (Ouyang et al., 2012a). Previously validated targets of miR-181 include the ER stress protein GRP78 (Ouyang et al., 2012b) and three antiapoptotic proteins, BCL2, MCL1 (Ouyang et al., 2012a) and XIAP (Hutchison et al., 2013). While treatment before stroke provides evidence that anti-miR-181 can protect in acute stroke, treatment after stroke onset will be needed in most cases, as patients often present hours after stroke onset. Thus to assess potential translational relevance we tested the effect of post-ischemic treatment with miR-181a antagomir administered by intracerebroventricular (ICV) or intravenous (IV) injection in a transient focal cerebral ischemia model. We assessed both short term and long term outcomes with post-treatment, including rotarod neurobehavioral assessment.

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Materials and methods

miRNA-181a antagomir

miRNA-181a antagomir and a negative control (mismatched (MM)-miR-181a antagomir) were from Thermo Scientific (Hudson, New Hampshire, USA) and the sequences are:

Antagomir miR-181a (MAGWA-000005)
mA.∗.mC.∗.mU.mC.mA.mC.mC.mG.mA.mC.mA.mG.mC.mG.mU.mU.
mG.mA.mA.mU.∗.mG.∗.mU.∗.mU.∗.3'-Chl

MM - Antagomir miR-181a (MAGWA-00006)
mA.∗.mG.∗.mU.mC.mA.mG.mC.mG.mA.mG.mA.mG.mC.mC.mU.mU.
mG.mA.m U.mU.∗.mG.∗.mU.∗.mU.∗.3'-Chl

mN = 2′-O-Methyl nucleotide (N = A or C or G or U); ∗ = phosphorothioate linkage.

Reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR)

RT-qPCR for miRNA quantitation in the brain tissue was reported previously (Ouyang et al., 2012b). All materials were from Applied Biosystems (Foster City, CA). Total RNA was isolated with TRIzol®, then reverse transcription of equal amounts of RNA (200 ng) was performed using the TaqMan MicroRNA Reverse Transcription Kit and 1.3 mM dNTPs (with dTTP), 50 U reverse transcriptase, 10 U RNase inhibitor, and specific miRNA reverse transcriptase primers at 16 °C for 30 min, 42 °C for 30 min, and 85 °C for 5 min. PCR reactions were then conducted using the TaqMan® MicroRNA Assay Kit at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Each reaction contained 0.75 μl of the RT reaction product, 5 μl TaqMan 2× Universal PCR Master Mix in a total volume of 10 μl using the 7900HT Fast Real-Time PCR System (Life Technologies, South San Francisco, CA, USA). Predesigned primer/probes for miRNAs and mouse U6 were from Applied Biosystems. The expression of miR-181a was normalized using U6 as the internal control. Measurements were normalized to U6 (ΔCt) and comparisons calculated as the inverse log of the ΔΔCT to give the relative fold change for all miRNA levels (Livak and Schmittgen, 2001). Liu et al. have validated U6 as not changing in cerebral ischemia (Liu et al., 2010). The PCR experiments were repeated 3 times, each using separate sets of samples.

Transient focal cerebral ischemia

All experimental protocols using animals were performed according to protocols approved by the Stanford University Animal Care and Use Committee and in accordance with the NIH guide for the care and use of laboratory animals. Adult male CB57/B6 mice (25–30 g from Charles River) were anesthetized with 2% isoflurane in balance O_2 by facemask and focal cerebral ischemia was produced by 1 h of middle cerebral artery occlusion (MCAO) with a silicone-coated 6-monofilament (Doccol Co, Redlands, CA, USA) followed by reperfusion as described before (Ouyang et al., 2012b). Sham-operated mice underwent an identical procedure, without inserting the suture but tying off the ipsilateral external carotid artery. Rectal temperature was maintained at 37 ± 0.5 °C controlled by a Homeothermic blanket control unit (Harvard Apparatus, Holliston, MA, USA). Temperature and respiratory rate were monitored continuously. Mice were randomized to surgery or sham, and mice with no evidence of acute neurological deficit or with evidence of hemorrhage were excluded from analysis. A total of 220 mice were subjected to sham or MCAO surgery, 18 were excluded from analysis, 12 animals died prior to day 7, 4 had evidence of hemorrhage, and 2 had no evidence of neurological deficit acutely. After different durations of reperfusion, mice were deeply anesthetized, and brains were removed after transcardiac perfusion with ice cold phosphate buffered saline (PBS) for RT-qPCR, Western blot analysis, or first with PBS and then 4% paraformaldehyde in PBS for immunohistochemistry.

Intracerebroventricular infusion (ICV) and intravenous injection (IV) of 181a antagomir

Two hours after MCAO miR-181a antagomir or control mismatch antagomir (MM) was injected intracerebroventricularly as shown previously (Ouyang et al., 2012b). Mice were anesthetized and placed in a stereotaxic frame with a mouse head holder. The brain infusion cannula was stereotaxically placed into the left lateral ventricle (bregma: −0.58 mm; dorsoventral: 2.1 mm; lateral: 1.2 mm) *via* a burr hole (Xiong et al., 2011) and affixed to the skull. Antagomir (3 pmol/g in 2 μl final volume) or MM (same amount) was mixed with the cationic lipid DOTAP (4 μl; Roche Applied Science, San Francisco, CA). After mixing for 5 s and incubating at 37 °C for 15 min, the mixture (total 6 μl) was infused into the left lateral cerebral ventricle over 20 min. After that the bone wound was closed with bone wax. For intravenous infusion, 1 h after MCAO miR-181a antagomir (30 pmol/g) or control (MM) in sterile saline (100 μl) was administered into the internal jugular vein. The schematic timecourse is shown in Fig. 1A.

Determination of infarct volume and neurological status

Cerebral infarction volume was determined after 2,3,5-triphenyltetrazolium chloride (TTC, Sigma, T8877, St. Louis, MO 63103, USA) staining of brains harvested at 48 h reperfusion. Four sections/mouse were analyzed by a blinded observer, and corrected for edema using Adobe Photoshop CS3 as described previously (Han et al., 2009). Neurological status was assessed by a neurologic deficit score at 24 h reperfusion (Xiong et al., 2011). Neurological deficit score: 0 — no observable neurological deficits, 1 — failure to extend right forepaw, 2 — circling to the right, 3 — falling to the right, and 4 — cannot walk spontaneously.

Rotarod test

Motor coordination and learning were assessed with the accelerating rotarod (SD Instruments, San Diego, CA). Beginning on the first day of training and continuing every other day for a total of 9 training days each mouse was placed on the 2.75 cm diameter rod with rotation speed increasing from 5 to 10 rpm over 5 min. The time in which the mouse was able to stay on the rotating rod before falling was determined up to a maximum duration of 300 s. The test was repeated three times each day for each mouse, and the scores were averaged for each day. Any mouse not able to achieve at least a 200 second average on the rotarod after 9 days of training was excluded from the study. Rotarod testing was performed 3 days, and 1–4 weeks after surgery.

Immunohistochemistry

Ischemic or sham-operated mice were deeply anesthetized and perfused with cold 0.9% saline, followed by 4% paraformaldehyde in phosphate-buffered saline (PBS) (pH 7.4) 24 or 48 h after MCAO. The brains were kept in 4% paraformaldehyde in PBS (pH 7.4) for 3 days then cut into 50 μm sections with a vibratome (VT1000S, Leica Microsystems, Wetzlar, Germany). Sections were immunostained using Polyclonal Rabbit Anti-Human Myeloperoxidase (MPO dilution 1:600, Cat # A03978 Dako, Carpinteria, CA, USA) to detect infiltrating neutrophils, polyclonal antibody to Iba1 (Iba1, dilution 1:200, Cat # 019-19741 Wako Chemicals, Richmond, VA, USA) to identify activated microglia/macrophages, and X-linked inhibitor of apoptosis (XIAP) antibody (1:500 dilution, catalog #3331, ProSci, Poway, CA, USA).

After staining, evaluation was done in a fixed 1 mm × 1 mm area of the penumbra. The penumbra was defined as the region of generally morphologically normal cells surrounding the core (see Fig. 1B). To quantify levels of fluorescence intensity 4 brain sections for each of