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siRNA-mediated silence of protease-activated receptor-1 minimizes ischemic injury of cerebral cortex through HSP70 and MAP2

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ABSTRACT

Cerebral ischemic stroke is a prevalent disease in senior individuals. The anticoagulation and thrombolysis to recover blood supply as well as the diminution of neural excitotoxicity to protect brain cells have not shown to fully improve stroke patients. The comprehensive mechanisms and medication specificity remain to be addressed. The silence of specific mRNAs by RNA interference provides revenues for such goals. We examined whether the silence of protease-activated receptor-1 (PAR-1) by siRNA protects brain tissues from ischemic injury. In three groups of Wistar rats, their lateral ventricles received the injections of lentiviral vectors carrying siRNA for PAR1, small RNA in mismatching PAR1 or saline. A week after the injections, these rats were treated by one side of middle cerebral artery occlusion (MCAO). The scores of neurological deficits, the volume of ischemic infarction and the expressions of PAR-1, HSP-70 and MAP-2 were measured in 24 h of MCAO. Our results show that the silence of PAR-1 significantly reduces neurological deficits and infarction volume, as well as elevates HSP-70 and MAP-2 expressions. Thus, the knock-down of PAR1 minimizes the ischemic impairments of cerebral cortex via HSP70 and MAP-2 pathways.

1. Introduction

The therapies of ischemic stroke, i.e., anticoagulation, thrombolysis and neuronal protection have not shown substantially to improve stroke patients [1–5]. Specific molecular targets for its medication remain to be developed. Protease-activated receptor 1 (PAR-1) activated by thrombin plays a central role in coagulation cascade [6–8], and is widely distributed on the surfaces of brain cells [9–12]. Recent studies demonstrate that the PAR-1 protein is up-regulated during ischemia in hippocampus [9,13,14], and PAR-1 antagonist may protect cerebral tissues from ischemic injury and infarction [13,15,16]. Thus, PAR-1 is likely a potential therapeutic target for ischemic stroke.

RNA interference through micro-RNA is an evolutionarily conserved process of gene silence during post-transcription [17–23]. A delivery of small interference RNA (siRNA) built by chemical and biological ways into cells has been believed to specifically knock-down the designed genes [24–28]. We intend to examine whether the silence of PAR-1 by siRNA prevents the ischemic impairment of brain tissues. After identifying the effectiveness of siRNA on silencing PAR-1 genes, we examined the score of neurological deficits and the volume of ischemic impairment, as well as the expressions of heat shock protein-70 (HSP-70) and microtubule-associated protein-2 (MAP-2), two proteins in the protection of brain cells against ischemic stroke [29–31], to merit the outcome of PAR-1 knock-down to ischemia.

2. Materials and methods

2.1. Animals and surgery

The entire procedures were approved by Institutional Animal Care and Use Committee (ACUC) in Harbin Heilongjiang, China. Eighty-four healthy male Wistar rats (220–280 g), from the Institute of Laboratory Animal in Chinese Academy of Medical Science, were randomly divided into three groups, twenty-eight in each group. Lentivirus-carried (LV) siRNA-PAR1 (experimental group), LV-misRNA-PAR1 (negative control) and 0.9% NaCl (naïve control) were injected into the rats' lateral ventricle in these three groups, respectively. The amount of LV particles was the multiplication of 10^8/μl and 10 μl. Seven days after the injection, the rats were treated with middle cerebral artery occlusion (MCAO).

The surgical operations for the MCAO were performed based on the technique described by Zea-Longa [32,33]. The rats were anesthetized by the intraperitoneal injection of 4% sodium pentobarbital (40 mg/kg). During MCAO operations, a long nylon suture was inserted into the left

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internal carotid artery for 2 h. To completely block blood flow into MCA, an intraluminal suture was introduced via the extracranial ICA. The detailed procedures are given below.

The left side of CCA was exposed by midline incision under an operating microscope. A retractor was positioned between the digastric and sternomastoid muscles, and the omohyoid muscle was divided. An ICA was isolated fully from an adjacent vagus nerve. Further dissection exposed glossopharyngeal nerve at the origin of pterygopalatine artery. The extracranial branch of the ICA was ligated with 7–0 nylon suture close to its origin. At this point, the ICA is the only remaining extracranial branch of the CCA. Subsequently, a 6–0 silk suture was tied loosely around the mobilized ECA stump, and a curved microvascular clip was placed across both the CCA and ICA adjacent to the ECA origin. A 5 cm length of 4–0 monofilament nylon suture, whose tip were smoothed by flame heating, was inserted into the ECA lumen via a puncture on one of terminal branches of the ECA. The silk suture around the ECA stump was tightened around the intraluminal nylon suture to prevent bleeding, and microvascular clip was removed. The nylon suture was then gently advanced from the ECA to the ICA lumen; the position of the suture within the ICA lumen could be seen when it reached the base of the skull. After this nylon suture had been inserted into the ECA stump, resistance was felt and a slight curving of the suture or stretching of the ICA was observed, indicating that the blunted tip of the suture had passed into the MCA origin and reached the proximal segment of anterior cerebral artery (ACA), which has a smaller diameter. At this point, the intraluminal suture has blocked the origin of the MCA, and occluded all sources of blood flow from the ICA, ACA and posterior cerebral artery (PCA). The incision was closed, leaving 1 cm of the nylon suture out such that it could be withdrawn to allow reperfusion. The restoration of MCA blood flow can be done by pulling the suture back until the resistance was felt, indicating that the tip had cleared the ACA-ICA lumen and was in the ECA stump, and then trimmed.

The rats in three groups were randomly selected into two groups that were decapitated at 24 or 72 h after MCAO, and their brain tissues were harvested to examine the injury of ischemia and the expressions of mRNA and proteins. It is noteworthy that all of the rats were evaluated and scored by neurological deficits (see below) immediately before decapitation.

2.2. The design of siRNA design and the construction of lentiviral vectors

Based on the sequence of protease-activated receptor 1 (PAR-1) mRNA, we designed the hairpin format of small interference RNAs (siRNA) by using the guidelines in the publications [32–36] and a public software Ambion (Ambion Inc., Austin, TX, USA) [37,38]. In the design, multiple interference sites of PAR-1 mRNA were made to search an optimal sequence of siRNA for PAR-1 knock-down. After the experimental evaluation and computational modeling of cultured cells, we selected an optimal sequence of siRNA for PAR-1 knock-down (called as siRNA-PAR1), which was "GTCTGCTACACGTCCATCA". In the synthesis of DNA oligo that contains siRNA sequence, the limited endogenous-enzyme sites were added into both sides of oligo. After cutting by the enzymes, this interference sequence was inserted into the backbone plasmid of lentivirus. This plasmid then was amplified, cloned and identified in bacterial sensory cells. The positive clones including siRNA-PAR1 were collected. The selected plasmids were purified and concentrated. The condensed plasmids with other packing plasmids were then cotransfected by transient calcium phosphate to pack lentiviruses in 293T cells. Lentiviral vectors were harvested and concentrated to a level of 10^8.

As a high specificity of siRNA [39–43], the mismatch mutations in its stem sequences abolish the function of silencing mRNA. Based on this principle, we introduced a negative control siRNA that mismatches the sequence of PAR-1 mRNA (called as misRNA for PAR-1) and has no effect on silencing PAR-1 mRNA. All of the reagents for molecular biology were purchased from GeneChem Co., Ltd in Shanghai.

2.3. Scores of neurological deficits

The neurological status was evaluated and scored carefully 24 and 72 h after MCAO, or immediately before the decapitation. The scales from zero to four were used to assess the effects of MCAO on neurological behavior [32,33]. The rats were suspended 1 m above the floor by gently holing their tail, and the degree of their forelimb flexion was monitored. The rats that extended both forelimbs toward the floor and had no other neurological deficits were assigned grade 0. The rats without full flexion in their forelimb contralateral to MCAO were graded as 1. The rats that showed consistently reduced resistance to lateral push toward the paretic side as well as circling behavior were graded as 2. The rats that circled toward the paretic side consistently were graded as 3. The rats with a depressed level of consciousness and unresponsiveness to stimulation were graded as 4. The neurological testing was performed by a single observer who was blinded to know the assignments of three groups.

2.4. Triphenyltetrazolium chloride (TTC) staining

The rats were decapitated 24 h and 72 h after MCAO and immediately after the neurological test, respectively. The brain tissues were removed rapidly, and the coronal sections between 2.5 mm and 4.5 mm from the frontal end were sliced within 3 min of their death. The sections were immersed in 4% 2,3,5-triphenyltetrazolium chloride (TTC) at 37 °C for 30 min. The areas of ischemic infarction with TTC staining appeared gray, whereas the areas in the absence of infarction were stained as red. The sizes of the infarct areas and whole sections were analyzed by Image-Pro Plus 6.0. Infarction volume was calculated based on the formula $V = t(A_i1 + A_i2 + \ldots + A_in)/t(A_w1 + A_w2 + \ldots A_wn)$, in which t is the slice thickness, n is the number of slices, A_i is the size of the infarction area in a slice, and A_w is the size of the whole coronal section. As we examined infarction in the coronal section, the infarction volume was expressed as a ratio of infarct volume to coronal section volume in the whole brain.

2.5. Western blot and immunohistochemistry

The samples were obtained from the MCAO side of cortical tissues including hippocampus. Protein extraction, SDS-PAGE and western-blot were conducted based on the manufacturer's instructions [34,35]. The concentration of proteins was 2 μg/μl. They were separated on an 8% SDS-PAGE gel and transferred onto PVDF membrane. The polyclonal antibodies of anti-PAR1 and anti-β-actin [F-1804 (Sigma, 1: 4000) and KC-5G4 (KangChen, 1:4000)] were the primary antibodies for PAR-1 and β-actin, respectively. SC-2005 (Santa-Cruz, 1:4000) was the secondary antibody in HRP-conjugated. The total protein content was determined with a BCA protein assay kit (Bio-Rad, Mississauga, ON, Canada). The intensities of protein bands were measured by the ECL detection system (Amersham), and were quantified by using an Odyssey software (v2.1 LI-COR), in which band intensities (area × OD) were measured for each of the groups and normalized by ß-actin band intensities.

Immunohistochemistry for HSP-70 and MAP-2: tissue blocks from the ischemic area of MCAO were sliced in cross section at 20 μm by a freezing microtome. The sections were washed by PBS for three times and stained by immunohistochemistry. They were incubated in polyclonal antibodies of anti-HSP70 or anti-MAP2 (Abcam, USA) at 4 °C with shaking for 24 h, and subsequently incubated in HRP-conjugated anti-rabbit antibodies (Abcam, USA). The distributions of HSP70 and MAP2 (dark brown in color) were observed under conventional optical microscope. Five fields were randomly selected in the penumbra around the infarct areas. Their photos were taken by using Mike Audi