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Expression of RGMb in brain tissue of MCAO rats and its relationship with axonal regeneration

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ABSTRACT

Objective: To investigate the changes in the expression of repulsive guidance molecule b (RGMb) in brain tissue of rats with ischemic cerebral infarction and determine its relationship with axonal regeneration, synapse remodeling and magnetic resonance imaging (MRI) parameters with magnetic resonance diffusion tensor imaging as the dynamic continuous monitoring method *in vivo*, so as to explore the pathophysiological mechanism of the occurrence, development and prognosis of cerebral infarction.

Methods: Ninety Sprague-Dawley (SD) rats were randomly divided into six groups, namely control group, middle cerebral artery occlusion (MCAO) 12-h group, MCAO 24-h group, MCAO 48-h group, MCAO 7-day group and MCAO 10-day group, each of 15 animals. Rats were examined by head MRI at corresponding time points, followed by measurement of apparent diffusion coefficient (ADC) and fractional anisotropy (FA) values. Subsequently, brain tissues were taken to detect the expression of RGMb, axonal regeneration and synapse remodeling.

Results: After infarction, ADC and FA values of the infarcted core area were lower in rats of each group compared to those in rats of normal group ($P < 0.05$), which was lowest at 12 h. The positive expressions of RGMb and synaptophysin were continuously increased from the 12th hour after operation, which reached a peak at 48 h; while axonas was gradually reduced after operation in each group, which revealed the most obvious damage in the MCAO 24-h group. The protein expression of RGMb was negatively correlated with MRI parameters and axon growth.

Conclusion: After supratentorial cerebral infarction, the expressions of RGMb and synaptophysin were up-regulated in rats, neurofilament protein (NF-200) expression was decreased, and MRI parameters (ADC and FA values) were reduced, indicating that RGMb protein may be involved in the regeneration and remodeling of axons and synapses, and exert an important role in pathophysiological processes such as nerve regeneration disturbance and neuron apoptosis after cerebral ischemia injury. *In vivo* MRI can be a noninvasive technique to monitor the areas of cerebral infarction and the recovery of neurological function.

1. Introduction

Ischemic cerebrovascular disease (ICVD) is the most common disease that threatens human health and quality of life at present. Although this illness can be effectively controlled in the majority of patients *via* clinical standard treatment, and a certain degree of recovery of neurological function has been achieved, 70%–80% of these patients continue to have different degrees of sequelae of cerebral infarction [1]. Therefore, the effective rescue of ischemic damaged neurons and central nerve regeneration have become the key to neurological rehabilitation after cerebral infarction. It is well-known that peripheral nerves are repairable. However, an actual study has revealed that peripheral nerve regeneration is limited after damage of the central nervous system (CNS) in adult mammals, and scholars consider that the cause is the nerve regeneration inhibitory protein in CNS [2,3].

After cerebral infarction, hypoxia, ischemic injury, as well as apoptosis and necrosis, occur in neurons; and multiple factors are involved in the complex process through positive or negative regulation and interaction *via* their specific kinase pathways. Repulsive guidance molecule (RGM) has been newly found [4] to encode three kinds of isomeric protein products in vertebrates, known as RGMa, RGMb and RGMc [5]. An *in vitro* experiment demonstrated that axon growth can be inhibited by RGMb can inhibit in the central nerve and cerebellar granule cells in neonatal rats by activating the Rho kinase pathway [6]. In the adult rat model of cerebral ischemia reperfusion, the expression of RGMb was distinctly up-regulated after 48 h. Studies have displayed

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that RGMb is mainly expressed in the CNS of young rats and participates in the development of the nervous system, which is decreased with growth and development. When reaching adulthood, once the CNS is damaged (re-expression or over-expression), inhibition of nerve regeneration mainly occurs, followed by the participation in apoptosis [7–9]. The present study aimed to observe the changes in expression of RGMb in the core area of brain tissues in rats, and analyze the relationship of RGMb with axonal regeneration, synapse remodeling and magnetic resonance imaging (MRI) parameters using magnetic resonance diffusion tensor imaging (DTI) as the *in vivo* dynamic continuous monitoring method, combined with traditional pathology and advanced imaging technology, in order to further explore the pathophysiological mechanism of the occurrence, development and prognosis of cerebral infarction, and search for a new target of clinical treatment.

2. Materials and methods

2.1. Animals and experimental groups

Sprague-Dawley (SD) rats (weight: 260–320 g, mean: 299.2 ± 8.6 g) were provided by the Experimental Animal Center of Henan Province. Ninety rats were randomly divided into six groups (n = 15, each group): control group, middle cerebral artery occlusion (MCAO) 12-h group, MCAO 24-h group, MCAO 48-h group, MCAO 7-day group, MCAO 10-day group, in order to observe indexes in the acute and sub-acute phases. Rats were scanned by MRI according to the indicated time points above, and were sacrificed for immunohistochemistry staining and reverse-transcription polymerase chain reaction (RT-PCR) after MRI scanning to assay the RGMb expression in the left infarction core. Furthermore, the relationship between MRI parameters and molecular biological markers was analyzed to evaluate the impact of RGMb to axonal regeneration and synapse remodeling after cerebral infarction.

Throughout these experiments, the observers were blind to the experimental conditions.

2.2. Preparation of the focal cerebral ischemia model

MCAO was performed by intraluminal nylon filament intrusion in accordance with Longa's method [10]. Briefly, after the rats were anesthetized with 3.5% chloral hydrate (350 mg/kg), a longitudinal midline incision was made on the neck, and the left common carotid artery (CCA), external carotid artery (ECA) and internal carotid artery (ICA) were exposed. Then, the superior thyroid and occipital arteries were cut from the ECA by electrocautery, and the extremity of the ECA was ligated with sutures. Furthermore, branches between the ECA and ICA were also coagulated. In order to prevent hemorrhage, micro aneurysm clips were placed at the both ICA and ECA. After an incision was made distal to the ECA, the head end of a nylon filament suture coated with paraffin wax was inserted into the ICA. Before the micro aneurysm clip was removed from the ICA, the ECA was ligated with thread to prevent hemorrhage. The suture in the ICA was continuously advanced approximately 18-20 mm beyond the carotid bifurcation until mild resistance was felt.

Animals in the control group were treated similarly, except that the nylon filament was not advanced to the origin of the middle cerebral artery (MCA). Animals in other groups were induced to the focal cerebral ischemia model according to the above procedure.

2.3. MRI scan

The examination was conducted through Signa HDxt 3.0T MRI scanner and an eight-channel rat specific coil (Chenguang, Shanghai) provided by GE (USA). All experimental animals underwent coronal T1-weighted imaging and T2-weighted imaging, sagittal T2-weighted imaging, diffusion-weighted imaging (DWI) and DTI sequence scans, with the scan parameters shown in Table 1.

Table 1
The MRI scan parameters.

Sequence	TR/TE	FOV	NEX	Matrix
Fast spin echo (FSE)-T1WI	360/23.3 ms	70 × 70 mm	4.00	320 × 256
FSE-T2WI	2300/115.3 ms	70 × 70 mm	4.00	256 × 256
Single-shot SE/EPI-DWI	2350/78.9 ms	110 × 110 mm	4.00	96 × 96
DTI	2500/92.2 ms	110 × 110 mm	4.00	128 × 128

A post-processing workstation provided by GE (USA) was used for the post-processing of images and parameter statistics. Each site was repeatedly measured three times, followed by selection of the mean value.

2.4. Samples collection

All animals were sacrificed after the MRI scan. After the brains were removed, the left infarction core was dissected and preserved in liquid nitrogen for detection of the mRNA and protein expression of RGMb. In order to perform immunohistochemistry staining, the other animals were transcardially perfused with 200 ml of 0.9% saline, followed by perfusion with 250 ml of 0.1 M of phosphate buffer containing 4% paraformaldehyde. The brains were unloaded and post-fixed in 4% paraformaldehyde for 24 h, imbedded in paraffin, and coronally sectioned at approximately 6–8 μm.

2.5. RT-PCR analysis

Total RNA was extracted from the infarction core using Trizol reagent (Takara) according to manufacturer's instructions, and dissolved in RNase-free water. For each sample, the integrity of the RNA was verified by the detection of the 18S and 28S bands after agarose-formaldehyde gel electrophoresis. The purity of the total RNA was confirmed by optical density measurements at 260 and 280 nm. All RNA samples were stored at − 80 °C before reverse transcription. Total RNA was reversely transcribed (RT) into cDNA using a reverse transcriptase kit (Takara), according to manufacturer's instructions.

RT-PCR was carried out to determine the mRNA levels of RGMb in each group. The primers were designed using the Prime Premier software according to the Gene Bank sequences. The primer sequences were as follows: sense 5′-TGCCAACTTTAVTGCTGC-3′ and antisense 5′-ACAGGCAAATCCCTACATC-3′ for RGMb (product size 117 bp); sense 5′-CAAGGTCATCCATGACAACTTTG-3′ and antisense 5′-GTCCACCACCCTGTTGCTGTAG-3′ for GAPDH (product size 496 bp).

PCR reaction conditions: 94 °C denaturation for 5 min, one cycle, 94 °C denaturation for 30 s, 60 °C annealing for 30 s, 72 °C extended for 45 s, a total of 33 cycles, 72 °C extended for five minutes.

A 5 μl amplification product of PCR was taken and treated by electrophoresis on 2% agarose gel. The voltage was generally set to 70–80 V. After electrophoresis, the gel was removed and placed under an ultraviolet lamp to observe and photograph the results. The results were quantitatively analyzed using Quantity One-4.4.0 software. The ratio of the target gene mRNA to the average optical density of the internal reference band was used to represent the relative expression of the target gene mRNA.

2.6. Immunohistochemistry

The immunohistochemical staining of horseradish peroxidase (HRP)-labeled streptavidin (SP) was used to detect the expression of RGMb, neurofilament protein (NF-200) and synaptophysin in the infarcted core area of the brain tissue obtained from rats. The paraffin wax was conventionally cut into sections and placed in an oven at 60 °C