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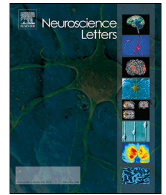
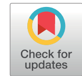


Research article

Effects of long-term rapamycin treatment on glial scar formation after cryogenic traumatic brain injury in mice



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ABSTRACT

Glial scar impedes axon regeneration and functional recovery following traumatic brain injury (TBI). Although it has been shown that rapamycin (a specific inhibitor of mammalian target of rapamycin) can reduce astrocyte reactivation in the early stage of TBI, its effect on glial scar formation has not been characterized in TBI and other acute brain injury models. To test this, ICR mice received daily administration of rapamycin (0.5 or 1.5 mg/kg, i.p.) beginning at 1 h after cryogenic TBI (cTBI). The results showed that at 3 d post-injury, 1.5 mg/kg rapamycin increased cTBI-induced motor functional deficits and infarct size, and attenuated astrocyte reactivation in the ipsilateral cortex, while 0.5 mg/kg rapamycin did not worsen brain damage and only slightly attenuated astrocyte reactivation. Furthermore, at 7 and 14 d after cTBI, 0.5 mg/kg rapamycin group showed a better motor functional performance than cTBI group. At 14 d post-injury, 0.5 mg/kg rapamycin significantly reduced the area and thickness of glial scar and chondroitin sulfate proteoglycan expression, accompanied by decreased expression of p-S6 and enhanced expression of growth associated protein 43 (an axon regeneration marker) in the region of glial scar. Our data suggest that long-term treatment with rapamycin can inhibit glial scar formation after cTBI, which may be involved in the mechanisms of increased axon regeneration and improved neurological functional recovery, and low-dose rapamycin may be more beneficial for such a therapy.

1. Introduction

Traumatic brain injury (TBI) is the primary cause of death and disability in population. It causes primary mechanical injury and long-lasting secondary injury. A series of pathological processes are responsible for the neuronal death observed in the secondary injury, such as glutamate excitotoxicity, oxidative stress, inflammation and gliosis [11]. There are no available treatments for the patients with enduring deficits induced by such injury. Although TBI damage can be devastating, many patients undergo at least a partial spontaneous recovery of brain function, which is related to neuroreparative processes, such as neurogenesis, axonal sprouting and neural network reconstruction [11]. Therefore, how to further augment the functional recovery is very crucial for the cure of TBI, which deserves further investigation.

Astrocytes play an important role in neuronal pathophysiological processes following diverse brain injuries, including TBI. It can be reactivated within 24 h after TBI, characterized by hypertrophic morphology, enhanced expression of intermediate filaments such as glial fibrillary acidic protein (GFAP), and increased proliferation [10,25]. In the early stage of TBI, reactive astrocytes are beneficial for preserving neural tissue and restricting inflammation [5,29]. However, in the chronic stage of TBI, the glial scar, a barrier mainly consisting of astrocytes, may impede neuroregeneration and neurogenesis. Recent researches have shown that inhibition of glial scar may facilitate neurorepair and neurological functional recovery [9,21,30]. Therefore, inhibiting the glial scar formation in the chronic stage without affecting the appropriate astrocyte reactivation in the early stage might be a potential therapeutic strategy for TBI.

Mammalian target of rapamycin (mTOR) is a rapamycin-sensitive serine/threonine protein kinase [18], which can be specifically inhibited by rapamycin, a kind of macrolide antibiotic [12]. Some studies show that mTOR inhibition prevented neuronal damage in the early

Abbreviations: CSPGs, chondroitin sulfate proteoglycans; cTBI, cryogenic traumatic brain injury; GAP-43, growth associated protein 43; GFAP, glial fibrillary acidic protein; mTOR, mammalian target of rapamycin; PTEN, phosphatase and tensin homolog; SCI, spinal cord injury; TBI, traumatic brain injury

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stage of TBI [6,10,12,19,24], while others suggest that increased mTOR signaling promoted neuroregeneration in the chronic stage of TBI [8]. Recently, series of evidences have manifested that mTOR signaling is also involved in astrocyte reactivation and glial scar formation in diverse central nervous system injuries. Inhibiting mTOR pathway by rapamycin or overexpression of phosphatase and tensin homolog (PTEN) attenuated glial scar formation and led to improved locomotor function after spinal cord injury (SCI) [1,23,28]. Deletion of mTOR in reactive astrocytes suppressed astrogliosis induced by temporal lobe epilepsy [26]. In the oxygen-glucose deprivation and reperfusion model, rapamycin suppressed astrocyte proliferation, migration and production of inflammatory mediators [3]. Recently, Nikolaeva et al. found that a single administration of rapamycin at 1 h after controlled cortical impact reduced astrogliosis but not the number of reactive astrocytes in the ipsilateral hippocampus at 24 h after injury [10]. However, in brain injury models including TBI, whether rapamycin can regulate glial scar formation is still unclear. Thus, we hypothesized that continual rapamycin treatment after cryogenic TBI (cTBI) could attenuate glial scar formation, thereby improving neuroregeneration and neurological functional recovery.

2. Materials and methods

2.1. Animals

Eight-week-old male adult ICR mice were obtained from the Experimental Animal Centre of Shanxi Medical University and housed under diurnal lighting conditions (12 h light/dark) at 22–24 °C with free access to food and water. All animal experimental procedures strictly conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Every effort was made to minimize the number of animals used and their suffering.

2.2. cTBI model

Procedures for cTBI were previously described [22]. Mice were anesthetized with isoflurane, and a copper probe (3 mm in diameter) pre-cooled by liquid nitrogen was pressed onto the skull (0.5 mm anterior and 2 mm right-lateral to bregma as center) by a force of 150 g for 30 s, resulting in motor and somatosensory cortical lesions. The cut skin was sutured, and then the mice were placed in a heated cage to wake and recover spontaneously.

2.3. Measurement of infarct volume

To quantify the infarct volume, the serial frozen coronal brain sections (20 μm thick) were stained with 1% toluidine blue. The infarct volume was calculated as the contralateral hemisphere volume (obtained by multiplying the sum of the areas of the contralateral hemisphere by the distance between the sections) minus non-injured ipsilateral hemisphere volume (obtained by multiplying the sum of the areas of the non-injured ipsilateral hemisphere by the distance between the sections).

2.4. Rapamycin treatment

Rapamycin was dissolved in absolute ethyl alcohol (10 mg/mL) as a stock solution. The final drug solution was diluted before injection and contained 4% absolute ethyl alcohol, 5% polyethylene glycol 400, 5% Tween 80, and 86% normal saline. Mice received the first injection of rapamycin (0.5 mg/kg or 1.5 mg/kg, i.p.) at 1 h after cTBI. Daily rapamycin treatment continued until the animals were sacrificed, and the mice in the control and cTBI groups were injected with the vehicle.

2.5. Rotarod test

Rotarod test is now widely used to identify motor coordination and locomotor deficits in rodents [20]. As previously described [20], two days prior to cTBI, each animal received training trails on a rolling rod (3 cm in diameter) to acclimate and gain a basal level of competence. At 1, 3, 7 and 14 d after cTBI, mice were placed on the rod, which constantly accelerated from 4 rpm to 40 rpm over 600 s, and the latency to fall was recorded. Each test was repeated for three times (10 min rest intervals) and the mean value of the latency to fall was used for analysis.

2.6. Beam walking test

Beam walking test provides a well-established method to assess the precise forelimb and hindlimb locomotor activity in rodents among various types of brain injury [20]. As previously described [20], two days prior to cTBI, the non-injury mice received training trails to autonomously traversed the beam (0.8 cm in diameter, 100 cm in length) without footslips. In test trails, the number of total footsteps and footslips of the left hindlimb in three repeated trials (5 min rest intervals) was recorded at 1, 3, 7 and 14 after cTBI. Data were shown as the percentage of footslips, which is defined as the number of left hindlimb footslips divided by the total number of footsteps and multiplied by 100%.

2.7. Immunofluorescence

The blocked slices (10 μm thick) were incubated with anti-GFAP conjugated to Cy3® antibody (1:400, Abcam, USA), rabbit anti-growth-associated protein 43 (GAP-43) antibody (1:200, Abcam, USA) or rabbit anti-p-S6 (Ser235/236) antibody (1:100, Abcam, USA) at 4 °C overnight. After extensive rinses, brain slices were subsequently incubated with goat anti-rabbit IgG secondary antibody (Alexa Fluor 488, 1:500, Thermo Fisher, USA) at 37 °C for 1 h. Finally, images were obtained using a fluorescence microscope (Olympus DP71, Japan) or a confocal microscope (Olympus FV1200, Japan). The number of GFAP-positive cells in four fields around the infarct core as well as the glial scar area and thickness were measured by Image J software. In the glial scar region, which is around infarct core with an intensive expression of GFAP, the IOD value of p-S6 per square micrometer among the three fields and the IOD value of GAP-43 per square millimeter among the six fields were analyzed by Image-Pro Plus 5.0 software.

2.8. Western blotting analysis

The prepared protein samples were separated on 8% or 10% SDS-PAGE gels and blotted onto PVDF membranes. The blocked membranes were incubated with antibodies against GFAP (1:250, Boster, China), chondroitin sulfate proteoglycans (CSPGs, CS56, 1:1000, Sigma, USA), p-mTOR (1:1000, Abcam, USA), mTOR (1:1000, Abcam, USA), p-S6 (1:2000, Cell signaling, USA) and GAPDH (1:6000, Bioworld, USA) at 4 °C overnight. After repeated washing, the membrane was incubated with HRP-conjugated goat anti-rabbit IgG (1:3000, Boster, China) or HRP-conjugated goat anti-mice IgG (1:1000, Boster, China) at room temperature for 1 h. Images were captured using Bio-Rad imaging system and band density was analyzed by the AlphaView software.

2.9. Statistical analysis

All data were collected and analyzed in a blinded manner. Five to six biological replicates and at least 2 times of technical replicates were made in western blotting. Five to eight biological replicates in three repeated trials were made in behavior test. Data are presented as mean ± S.E.M. One-way analysis of variance with least significant difference or Dunnett's T3 post hoc test (where equal variances were

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