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Role of fractalkine/CX3CR1 signaling pathway in the recovery of neurological function after early ischemic stroke in a rat model

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ABSTRACT

This study aims to explore the role of fractalkine/CX3C chemokine receptor 1 (CX3CR1) signaling pathway in the recovery of neurological functioning after an early ischemic stroke in rats. After establishment of permanent middle cerebral artery occlusion (pMCAO) models, 50 rats were divided into blank, sham, model, positive control and CX3CR1 inhibitor groups. Neurological impairment, walking and grip abilities, and cortical and hippocampal infarctions were evaluated by Zea Longa scoring criterion, beam-walking assay and grip strength test, and diffusion-weighted magnetic resonance imaging. qRT-PCR and Western blotting were performed to detect mRNA and protein expressions. ELISA was conducted to measure concentration of sFractalkine (sFkn), interleukin-1β (IL-1β) and TNF-α. The recovery rate of neurological functioning impairment and reduced walking and grip abilities was faster in the positive control and CX3CR1 inhibitor groups than the model group. The model, positive control and CX3CR1 inhibitor groups showed increased mRNA and protein expression of chemokine C-X3-C motif ligand 1 (CX3CL1) and CX3CR1, concentration of sFkn, IL-1β and TNF-α, and size of cortical and cerebral infarctions while decreased expression of NGF and BDNF compared with the blank and sham groups. Compared with the model group, the mRNA and protein expression of CX3CL1 and CX3CR1, concentration of sFkn, IL-1β and TNF-α, and size of cortical and cerebral infarctions decreased while expression of NGF and BDNF increased in the positive control and CX3CR1 inhibitor groups. Thus, the study suggests that inhibition of fractalkine/CX3CR1 signaling pathway promotes the recovery of neurological functioning after the occurrence of an early ischemic stroke.

1. Introduction

Strokes are a major cause of adult disability and death all over the world [1]. It is predicted that nearly 12 million people will die from stroke, 70 million people will suffer from stroke, and approximately over 200 million disabled lives be lost from stroke every year by 2030 [2]. Ischemic stroke is one of the most common forms of stroke, frequently caused by an acute vessel occlusion, results in in brain parenchymal oligemia or ischemia [3]. Ischemic stroke accounts for 85% of all stroke cases, and another 15% is hemorrhagic stroke [4]. Although the majority of ischemic strokes are not fatal, the long-term disability is a common consequence resulting in increasing economic burden [5]. Patients with ischemic strokes experience daily life depreciating dysfunctions, but clinical and technological advancements have been made for patients with ischemic stroke [6–8]. Presently, 3-n-butylphthalide (NBP) is considered to be a potential drug for treating ischemic stroke, acting on multiple pathophysiological processes [9]. However, the search for novel anti-ischemic stroke agents with higher potency remains trivial and a focus of intensive research [10].

Fractalkine, also called chemokine C-X3-C motif ligand 1 (CX3CL1) or neurotactin, is a CX3C chemokine expressed in neurons, endothelial cells, hepatocytes and vascular smooth muscle cells [11,12]. Being a unique chemokine, fractalkine is known to be a membrane-bound protein which regulates cell-to-cell communication and adhesion as well as cell recruitment and survival through binding to the CX3C chemokine receptor 1 (CX3CR1) [11,13,14]. In addition, fractalkine signals microglia by binding to only one receptor, CX3CR1, which later functions as an inhibitor of microglial activity [15,16]. There is an increasing interest in the role played by fractalkine in injuries concerning the central nervous system. For example, fractalkine mediates interactions between neurons and glial cells in the brain [12]. CX3CR1 is important for sustaining normal microglial activity in the brain [17], and fractalkine levels decrease immediately upon the occurrence of neuronal damage, thus decreasing CX3CR1 receptor signaling which results in the recruitment and activation of microglia [18]. On the basis of previous researches/

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studies, we hypothesize that fractalkine/CX3CR1 signaling pathway may be correlated with the recovery of neurological functioning in patients with early ischemic stroke.

We designed a series of experiments in this study to investigate the correlation between fractalkine/CX3CR1 signaling pathway and the recovery of neurological function after early ischemic stroke in a rat model.

2. Materials and methods

2.1. Ethics statement

All experimental procedures were conducted in accordance with the guidelines for the care and use of laboratory animals. All procedures were performed with the approval of the Institutional Animal Care and Use Committee. Efforts were made to minimize animal suffering and reduce the number of animals included in the experimented [19].

2.2. Establishment of permanent middle cerebral artery occlusion (pMCAO) rat model and animal grouping

A total of 50 male Sprague Dawley (SD) specific pathogen free (SPF) rats (weighing 200–250 g, aged 4–6 weeks) were acquired from the Hunan SLAC Jingda Laboratory Animal Co., Ltd. (Changsha, China). The acquired SD rats were kept at a temperature of 25–26 °C in 60–70% relative humidity conditions with lumpy fodder and easy access to tap water. The rat cage padding was replaced twice a week. All SD rats were maintained in a standard feeding room with similar fodder, water, light and temperature conditions. 20 rats were selected and equally divided into the blank group (undergoing no treatment) and sham group (without the blockage of the middle cerebral artery), and the residual 30 rats were prepared for the establishment of the pMCAO model in accordance with a previous research [20]. The SD rats were anesthetized using 2% pentobarbital sodium (40 mg/kg body weight, Sigma, St. Louis, MO, USA) injections (dosage of 350 μL/100 g) and pinned to the anatomical table in the supine position. The neck and surrounding muscles were separated after a median incision was made on the neck. Subsequently, the common carotid artery (CCA), the external carotid artery (ECA) and the internal carotid artery (ICA) of the right side were exposed and separated for further use. The ICA was clipped on the distal end using an arterial clamp, while the CCA and ECA were ligated on the proximal ends. A nylon rope (18–19 mm) was inserted into the CCA and ECA bifurcation following a small incision in order to make right middle cerebral artery occlusion and cerebral ischemia models. The redundant nylon rope was discarded, and the surface wound was ligated and sutured.

Three days after pMCAO model establishment, 24 SD rats with 1–3 scores (Zea Longa neurological score) were selected and randomly assigned into the following 3 groups (8 rats in each group): the model group, positive control group and fractalkine/CX3CR1 signaling pathway inhibition group (CX3CR1 inhibitor group). Rats in each group were administered various substances once a day in an intragastric or intraperitoneal manner between the 3 and 14th days post operation. Equal amounts of edible oil was administered to the rats in the blank, sham and model groups, and 70 mg/kg butylphthalide (NBP Pharmaceutical Co., Ltd., Shijiazhuang, China) was administered to the rats in the positive control group. 80 μg/kg CX3CR1 neutralizing antibody (Abcam Trading Company Ltd., Shanghai, China) was administered to the rats in the CX3CR1 inhibitor group via intraperitoneal injections.

2.3. Neurological function scoring

Once the rats were completely awake, the neurological function of rats in each group was graded according to criteria proposed by Zea Longa as follows [21]: 0 score, unimpaired neurological function; 1 score, rats demonstrated contralateral forelimb flexion and adduction upon being hung by their tails, i.e., slight neurological function impairment symptoms; 2 scores, rats walked in circles leaning towards the left or the paralyzed side, i.e. moderate neurological function impairment symptoms; 3 scores, rats found difficulty in walking and falling to the left side and falling to the contralateral side of focus, i.e. severe neurological function impairment symptoms; 4 scores, the rats showed decreased level of consciousness and no spontaneous activity.

2.4. Beam-walking assay

Rats were trained to walk on a narrow balance beam 5 days prior model establishment and were evaluations were made on the 3rd 7th, 10th and 14th days post operation. Next, the beam-walking scores were recorded. The beam was 2 cm wide, 1 cm thick, 120 cm long and 80 cm above the ground. The scoring standards were as follows [22]: 0 score, if the rats were unable to stay on the beam; 1 score, if the rats could stay on the beam but were unable to move; 2 scores, if the rats showed movement but fell off half way; 3 scores, if the rats showed movement but the operated hind legs slipped >50%; 4 scores, if the rats could pass the beam and did not slip more than once and <50%; 5 scores if the rats succeeded in crossing the beam and slipped once across the beam; 6 scores, if the rats succeeded in crossing the beam without slipping.

2.5. Grip strength test

Grip strength tests were conducted on the 3rd, 7th, 10th and 14th days post operation. During the tests, rats gripped the tension bar of the instrument using their forelimbs, and researchers held the tension plate using their right hands and tugged on the rat tails using their left hands. Next the researchers loosened their right hand grip and pulled back the rat body until the forelimbs slipped from the tension bar. Maximum tension values were recorded as effective data. Each rat was tested three times, and the mean ± standard deviation (SD) was analyzed and recorded.

2.6. Enzyme-linked immunosorbent assay (ELISA)

The concentration of sFractalkine (sFkn), interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) were measured on the 3rd, 7th, 10th and 14th days post operation using an ELISA kit (PeproTech, Princeton, NJ, USA), and all blood was serially sampled. The antibody was diluted to a concentration of 1 μg/mL using a coating buffer, added into a 96-well plate (100 μL/well), coated and cultured overnight at 4 °C. Next, the coating buffer was abandoned and the plate was washed three times, followed by incubation with a blocking buffer (150 μL/well) at room temperature for 1 h. After the plate was washed, diluted standard or serum samples (100 μL/well) were added for incubation at room temperature for 2 h, and the blank control group was established. The plate was washed again and supplemented with horseradish peroxidase (HRP)-labeled avidin (100 μL/well), followed by incubation at room temperature for 1 h. The Tetramethyl benzidine (TMB) substrate was added (100 μL/well) into the plate after washing in order to initiate the reaction at room temperature for 15 min. Later, the reaction was terminated by the addition of 2 M sulphuric acid (50 μL/well). The absorbance values were detected at a wave length of 450 nm using a microplate reader, and the concentration of factors (ng/mL) were calculated and recorded according to a standard curve.

2.7. Hematoxylin-eosin (HE) staining

The rats were executed in order to extract brain tissues after behavior observations were made on the 14th day post operation. The brain tissues were extracted and fixed with a 4% paraformaldehyde-phosphate buffer (pH = 7.4) for a duration of 24 h. Next, the brain tissues were dehydrated using gradient ethanol, transparentized by xylene,