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# Inhibition of chemokine-like factor 1 protects against focal cerebral ischemia through the promotion of energy metabolism and anti-apoptotic effect

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ABSTRACT

Chemokine-like factor 1 (CKLF1) is a novel C-C chemokine, and plays important roles in immune response and brain development. In previous study, we have found that the expression of CKLF1 increased after focal cerebral ischemia and inhibition of CKLF1 using antagonist C19 peptide protected against cerebral ischemia. However, few studies have focused on the role of CKLF1 on neuronal apoptosis. The objective of present study is to investigate the role of CKLF1 on neuronal apoptosis by applying anti-CKLF1 antibodies in rat focal cerebral ischemia and reperfusion model. Antibodies against CKLF1 was applied to the right cerebral ventricle immediately after transient middle cerebral artery occlusion (MCAO), and infarct volume, neurological score, glucose metabolism and apoptosis-related protein were measured. Treatment with anti-CKLF1 antibody decreased infarct volume and neurological score, and inhibited neuronal apoptosis in a dose-dependent manner at 24 h after reperfusion. Anti-CKLF1 antibody also reduced the level of phosphorylation of Akt (P-Akt), and led to decrease of pro-apoptotic protein Bcl-2 associated X protein (Bax) and increase of anti-apoptotic protein B cell lymphoma-2 protein (Bcl-2) and the ratio of Bcl-2/Bax, and inhibited caspase-3 at last. In addition, positron emission tomography (PET) indicated that anti-CKLF1 antibody increased glucose metabolism in ischemic hemisphere. These results suggest that CKLF1 is associated with neuronal apoptosis after cerebral ischemia and reperfusion. Neutralization of CKLF1 with antibodies shows neuroprotective effects against cerebral ischemia, which may be involved in inhibition of Akt pathway, regulation of apoptosis-related protein expression, and improvement glucose metabolism in ischemic hemisphere. Therefore, CKLF1 may be a novel target for the treatment of stroke.

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## 1. Introduction

Stroke is one of the leading causes of death and the principal cause of disability in the world. However, there is currently no effective treatment (Chacon et al., 2008). Ischemic stroke occurs when the normal blood supply to the brain is disrupted, thereby resulting in the activation of a series of functional impairment that eventually leads to neuronal death (Dirnagl et al., 1999).

Apoptosis is an important pathophysiological phenomenon in stroke. Cerebral ischemia triggers the pathological pathways of the ischemic cascade and ultimately causes irreversible neuronal injury in the ischemic core within minutes of the onset (Broughton et al., 2009). The apoptotic mechanisms are involved in excitotoxicity, oxidative stress and inflammatory reactions after

*Abbreviations:* CKLF1, chemokine-like factor 1; MCAO, middle cerebral artery occlusion; TTC, 2,3,5-triphenyltetrazolium chloride; $^{18}$F-FDG, $^{18}$F-Ffluorodeoxy-D-glucose; PET, positron emission tomography; P-Akt, phosphorylated Akt; Bcl-2, B cell lymphoma-2 protein; Bax, Bcl-2 associated X protein; PI3K, phosphatidylinositol 3 kinase.

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cerebral ischemia. Excitotoxicity and oxidative stress activate microglia and astrocytes and release cytokines, chemokines. These inflammatory mediators led to infiltration of inflammatory cells, mainly neutrophils, to the ischemic region. Infiltrated neutrophils themselves also secrete cytokines which cause a further activation of glial cells. These processes all result in neuronal cell death and aggravate the damage to the ischemic brain (Lakhan et al., 2009).

Cerebral ischemia and reperfusion causes an inflammatory response due to oxidative damage, which triggers stress signaling processes that eventually result in cell apoptosis and death. There are a number of chemical mediators and pathways involved in this process. The central mediators of cell activation and recruitment are chemokines, which are small polypeptides that are produced in elevated amounts at the ischemic injury site by the infiltration of circulating immune cells and activation of resident cells, including microglia, astrocytes, neurons and endothelial cells (Jaerve and Müller, 2012). Chemokines have been divided into four subfamilies based on the number and spacing of their conserved cysteine residues in their sequences, and are classified as the CXC, CC, C, and CX3C chemokines. Some studies have indicated that inhibition or deficiency of chemokines was associated with reduced injury (Huang et al., 2013; Sousa et al., 2013; Schuette-Nuetgen et al., 2012). Therefore, the immunomodulatory role of chemokines is now being translated into anti-inflammatory interventions to reduce the destruction arising from the infiltration of inflammatory cells and consequent secondary damage after injury (Lambertsen et al., 2012).

CKLF1 is a novel human cytokine of the cysteine–cysteine chemokine gene family, firstly discovered through isolation from phytohaemagglutinin-stimulated U937 cells. CKLF1 displays chemotatic activities in a wide spectrum of leukocytes. The expression of CKLF1 is upregulated in inflammation and autoimmune diseases (Li et al., 2006). Recent studies suggest that CKLF1 plays an important role in brain development (Wang et al., 2010, 2012).

In previous studies, we found that expression of CKLF1 increased after focal cerebral ischemia, and inhibition of CKLF1 protected against cerebral ischemia. (Kong et al., 2011, 2012). However, the neuroprotective mechanism is not clear. Some studies indicated that neuroprotective effect against cerebral ischemia/reperfusion injury was associated with anti-apoptotic mechanism. Prevention of apoptosis becomes a therapeutic strategy to preserve brain tissues and promote functional recovery (Vogel et al., 2007). Therefore, the purpose of the present study was to investigate the effect of CKLF on neuronal apoptosis in cerebral ischemia by applying anti-CKLF1 antibody.

## 2. Materials and methods

### 2.1. Materials

Anti-rat CKLF1 neutralizing antibody obtained from Peking University Center for Human Disease Genomics. Antibodies against Bcl-2, Bax, P-Akt and caspase-3 were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). HRP-conjugated secondary antibodies were from Pierce Biotechnology (Thermo Fisher Scientific, Rockford, IL, USA). All other chemicals and reagents were purchased from Sigma–Aldrich Company, unless otherwise specified.

### 2.2. Animals and ischemia protocol

Male Sprague–Dawley rats (age, 7 weeks; weight, 260–280 g) were supplied by Experimental Animal Center of Chinese Academy of Medical Sciences (Beijing China). All of the procedures were in accordance with the standards established in the Guide for the Care and Use of Laboratory Animals published by the Institute of Laboratory Animal Resources of the National Research Council (United States) and were approved by the Animal Care and Use Committee of the Peking Union Medical College and the Chinese Academy of Medical Sciences. The assessment of measuring infarct volume and scoring neurobehavioral outcome is blinded. All efforts were made to minimize animal suffering and to reduce the number of animals used.

MCAO was performed as reported previously (Kong et al., 2012). Briefly, under 10% chloral hydrate (4 ml/kg, intraperitoneal injection), a 4–0 nylon thread was inserted into the right common carotid artery and advanced until the origin of the right middle cerebral artery was occluded. After 60 min of the occlusion, the thread was removed to allow reperfusion and the animals were returned to their cages.

### 2.3. Drug Administration

To evaluate the contribution of CKLF1 to cerebral ischemia, 5 μl of polyclonal anti-rat CKLF1 neutralizing antibody (obtained from Peking University Center for Human Disease Genomics) in saline at dose of 0.1 μg, 0.5 μg or 1 μg that were produced in rabbits immunized with CKLF1 or normal rabbit immunoglobulin G (1 μg) was applied to the right cerebral ventricle immediately after reperfusion, with the needle left in place for 5 min thereafter. 5 μl of saline was injected in the vehicle group. The coordinates of the injection site were as follows: 0.8 mm posterior to the bregma, 1.5 mm lateral to the midline, and 3.5 mm depth from the dural surface, according to the atlas.

### 2.4. Neurological function

Neurological score was taken by Longa's 5-point scale at 24 h after reperfusion respectively (Longa et al., 1989). A score of 0: no neurological deficit; a score of 1: failure to extend left forepaw fully; a score of 2: circling to the left; a score of 3: falling to the left; and a score of 4: did not walk spontaneously and had a depressed level of consciousness. The animals without symptoms of neurological impairment or dying after the surgery were rejected and other rats were recruited.

### 2.5. Infarct volume analysis

At 24 h after reperfusion, the animals were anesthetized, and brains were removed and cut into 2-mm-thick slices, for a total of six slices per animal. The slices were immersed in a 1% solution of 2, 3, 5-triphenyltetrazolium chloride (TTC) in phosphate-buffered saline at 37 °C for 30 min and fixed in 4% phosphate buffered formalin. Images of the slices were obtained with a scanner and a computer. The infarct areas and the hemisphere areas of each slice were determined by Image J. The infarct areas on each slice were summed and multiplied by slice thickness to give the infarct volume. To compensate for edema and atrophy, the corrected volume was calculated using the following equation: percentage hemisphere lesion volume = ((total infarct volume − (right hemisphere volume − left hemisphere volume))/left hemisphere volume) × 100%.

### 2.6. Nissl staining

After 24 h of perfusion, 5 rats in each group were anesthetized with 10% chloral hydrate and perfused from heart with 200 ml of normal saline and 200 ml of 4% paraformaldehyde solution. Brains were dehydrated and embedded in paraffin, then sectioned at a thickness of 5 μm. Four paraffin sections from each rat were dewaxed, rehydrated and stained with 0.5% (m/v) Toluidine Blue for 15 min. The sections from Nissl staining were examined under

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