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Exogenous fractalkine enhances proliferation of endothelial cells, promotes migration of endothelial progenitor cells and improves neurological deficits in a rat model of ischemic stroke

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HIGHLIGHTS

- Fractalkine improves neurological deficits in ischemic stroke rats.
- Fractalkine enhances vascular density in the peri-infarct region.
- Fractalkine stimulates the proliferation of endothelial cells.
- Fractalkine induces endothelial progenitor cell homing to the peri-infarct area.

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ABSTRACT

Fractalkine/CX3CL1, also called neurotactin, has been described as an angiogenic agent, and its expression is up-regulated in the penumbra after ischemia. This study was conducted to investigate the neovascular potential of fractalkine on rat models of transient middle cerebral artery occlusion (MCAO). Rats receiving intracerebroventricular injections of fractalkine were found to have improved neurological deficits, reduced cerebral infarct size and increased neuron survival for both doses (100 ng and 1 μg). Fractalkine exerted angiogenic effects that showed dose-dependent higher vascular densities in the peri-infarct area. Furthermore, exogenous fractalkine increased the proliferation of endothelial cells in a dose-dependent manner and enhanced the migration of endothelial progenitor cells at the higher dose (1 μg) in ischemic penumbra. In conclusion, intracerebroventricular administration of fractalkine reduces ischemic damage by promoting neuroprotection and by inducing endothelial cell proliferation and endothelial progenitor cell migration, thereby enhancing neovascularization in the peri-infarct region.

1. Introduction

Stroke is a major cause of mortality and disability worldwide, and 80% of it is ischemic stroke [31]. Functional recovery can significantly improve the quality of life of stroke survivors. Interestingly, studies demonstrate that higher blood vessel counts in the penumbra correlate with better functional recovery after ischemic stroke [1,13,31,32]. Accumulating data now suggest that neovascularization facilitates highly coupled neurorestorative processes that consequently lead to attenuation of ischemic brain injury [34]. Therefore, the use of agents and manipulations to stimulate neovascularization that restores oxygen and nutrient supply to the cerebral infarct area and that provides neurotrophic compounds to newly generated neurons and neuroblasts may be a potential therapy for ischemic stroke [1,4,29,34].

Post-ischemic neovascularization consistently includes angiogenesis (sprouting of new capillaries from existing microvessels), vasculogenesis (blood vessel formation by migration and differentiation of endothelial precursor cells), and arteriogenesis (growth or maturation of collateral conduits with large enough diameters) [6,19,27,33]. It is a complex process that involves multiple mechanisms and numerous cytokines, such as vascular endothelial growth factor (VEGF), erythropoietin and chemokines [1,9].

Abbreviations: MCAO, middle cerebral artery occlusion; EPCs, endothelial progenitor cells; TTC, Triphenyltetrazolium chloride; ICA, internal carotid artery; CCA, common carotid artery; ECA, external carotid artery; PBS, phosphate buffered saline; mNSS, modified Neurological Severity Scores.

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Fractalkine, the sole member of the CX_3C chemokine family, is expressed constitutively in neurons and can be induced in microglia, astrocytes and vascular endothelial cells [11,17]. It is primarily described as being the recruiter for inflammatory immune cells in ischemic stroke [8,16]. Recently, it has been described as an angiogenic chemokine. Previous studies have shown that fractalkine induced endothelial cell migration and proliferation, endothelial progenitor cell (EPC) migration and tube-like structure formation in a concentration-dependent manner *in vitro*, and stimulated new blood vessel formation *in vivo* [7,14,15,21,25]. The studies also showed angiogenic effects in ischemic diseases. For example, in rat models of hindlimb ischemia, intra-muscular injection of exogenous fractalkine improved blood perfusion by inducing angiogenesis [14]. However, whether fractalkine can similarly enhance neovascularization and contribute to functional recovery following ischemic stroke has not been elucidated.

In the present study, we investigated the neovascular and neuroprotective action of fractalkine in the restoration of ischemic injury, focusing on analyzing the neovascularization through angiogenesis and vasculogenesis, as well as neurological deficits, brain infarct volume and neuron survival. We found that intracerebroventricular administration of fractalkine induced endothelial cell proliferation and EPC migration and, consequently, enhanced neovascularization in the peri-infarct region that in turn alleviated tissue damage. This is the first confirmation of a neovascular contribution of exogenous fractalkine in ischemic stroke.

2. Materials and methods

2.1. Materials

Recombinant rat fractalkine (chemokine domain) was purchased from R &D Systems, Inc. (Minneapolis, USA). Triphenyltetrazolium chloride (TTC) was obtained from Sigma–Aldrich (St. Louis, MO, USA). Rabbit anti-CD34 was purchased from Beijing Biosynthesis Biotechnology Co., Ltd. (Beijing, China); mouse anti-NeuN was obtained from Merck Millipore (Massachusetts, USA); other primary antibodies were purchased from Abcam (Cambridge, UK). All secondary antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, USA).

2.2. Animals and treatment

A total of 45 adult male Sprague-Dawley rats weighing 250–280 g were obtained from the Center for Experimental Animals of Sun Yat-Sen University (Guangzhou, China) and maintained at a specific pathogen-free housing facility. All procedures performed on these animals were approved by the Institutional Animal Ethical Committee at Sun Yat-Sen University and were in accordance with the Guidelines for the Care and Use of Laboratory Animals of the National Institute of Health (Publication No. 80-23, revised 1996).

Transient (90 min) right middle cerebral artery occlusions were induced as previously described [2,22]. Rats were anesthetized with 0.35 g/kg i.p. chloral hydrate, and then a MCAO monofilament, 0.26 mm in diameter (BEIJING SUNBIO BIOTECH Co., Ltd), was inserted into the internal carotid artery (ICA) through a small incision in the common carotid artery (CCA) after ligation of the external carotid artery (ECA) and the proximal CCA. For the sham-operated group ($n = 6$), the filament was not inserted into the artery. For the experimental groups ($n = 13$/group), 30 min after occlusion, the rats received injections stereotaxically with either: 5 μl phosphate-buffered saline (PBS) for the control group, 100 ng recombinant rat fractalkine in 5 μl PBS for the low dose group and 1 μg recombinant rat fractalkine in 5 μl PBS for the high dose group through a micro syringe (RWD Life Science Co., Ltd) into the right cerebral ventricle (0.8 mm posterior, 1.5 mm lateral to the bregma, and 4.0 mm below the dura). The needle was retained for 5 min after each injection, and dental cement was applied to the skull hole to prevent leakage of the injected solution and cerebrospinal fluid. One set of rats ($n = 5$/group) were euthanized 14 days after MCAO for TTC staining; the other set of rats ($n = 8$/group) were euthanized 14 days after MCAO for immunostaining.

2.3. Neurological functional tests

Modified Neurological Severity Scores (mNSS) including motor tests, sensory tests, bean balance tests, evaluation of absent reflexes and abnormal movements were performed before MCAO and at 1, 7, and 14 days after MCAO [12].

2.4. TTC staining

Fourteen days after MCAO, rats ($n = 5$/group) were perfused intracardially with saline. The brain tissue was removed, stowed at −80 °C for 8 min, and sliced into 2.0-mm-thick sections (six slices per rat) with brain matrices (RWD Life Science Co., Ltd). The brain slices were incubated in 2% TTC under no-light conditions for 20 min at 37 °C, and then transferred into 4% paraformaldehyde for fixation. The areas of each slice were obtained using a digital camera, and the volumes of infarction were calculated using a modified method that has been described previously [24,30].

2.5. Immunofluorescence staining for neuron and vessel counts

Fourteen days after MCAO, rats ($n = 8$/group) were perfused with saline and phosphate buffered (pH 7.4) 4% paraformaldehyde. Then, the brains were postfixed in 4% paraformaldehyde for 12 h, and gradient dehydrated in 20% and 30% sucrose. For immunostaining, a series of 10 μm-thick sections were cut from bregma −1 to +1 mm on a cryostat (CM1900; Leica, Heidelberg, Germany). Every 10th coronal section, for a total of 5 sections, was used for immunofluorescence staining, and the region of interest was defined in each section as a zone with 800 μm width and length in the peri-infarct region that is immediately outside the infarct zone as described previously [10]. To quantify cerebral neuron survival and blood vessel density, the brain sections were stained with mouse anti-NeuN (1:500) or mouse anti-CD31 (1:200), and conjugated with goat anti-mouse 488 (1:1000). All staining sections were also counterstained with 4′,6-diamidno-2-phenylindole dihydrochloride (DAPI) to assist the confirmation of cells. Fluorescence signals were captured with a microscope (BX51; Olympus) and the numbers of neurons and blood vessels were analyzed with Image-Pro Plus image analysis software in 8 non-overlapping fields (425 μm × 320 μm) (Media Cybernetics, Silver Spring, MD, USA) under 400× magnification.

2.6. Double immunofluorescence analysis of endothelial cell proliferation and EPC homing

To identify whether fractalkine could increase endothelial cell proliferation and EPC migration in perifocal fields, double immunofluorescence labeling for rabbit anti-Ki67 (1:100) with mouse anti-CD31 (1:200) or rabbit anti-CD34 (1:200) with mouse anti-KDR (anti-VEGF receptor 2, 1:100) conjugation with goat anti-mouse 488 (1:1000) and goat anti-rabbit 555 (1:1000) were performed. Images were acquired using fluorescent microscopy (BX51; Olympus) with a digital camera and the numbers of colocalized Ki67/CD31 or CD34/KDR immunopositive cells were