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# MCP-1-mediated activation of microglia promotes white matter lesions and cognitive deficits by chronic cerebral hypoperfusion in mice

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ABSTRACT

Microglia activation played a vital role in the pathogenesis of white matter lesions (WMLs) by chronic cerebral hypoperfusion. In addition, hypoxia induced up-regulated expression of MCP-1, promotes the activation of microglia. However, the role of MCP-1-mediated microglia activation in chronic cerebral ischemia is still unknown. To explore that, chronic cerebral hypoperfusion model was established by permanent stenosis of bilateral common carotid artery in mice. The activation of microglia and the related signal pathway p38MAPK/PKC in white matter, and working memory of mice were observed. We found that stenosis of common carotid arteries could induce MCP-1-mediated activation of microglia through p38MAPK/PKC pathway and white matter lesions. Taken together, our findings represent a novel mechanism of MCP-1 involved in activation of microglia and provide a novel therapeutical strategy for chronic cerebral hypoperfusion.

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## 1. Introduction

White matter lesions (WMLs) are involved in stroke and aging and stroke and contribute to the pathology of Binswanger disease, associated with subcortical vascular dementia (Targosz-Gajniak et al., 2009; Liu et al., 2008; Yoshizaki et al., 2008). These WM lesions are one of the factors for cognitive impairment and are mediated by chronic cerebral hypoperfusion (Nakaji et al., 2006; Fernando et al., 2006; Shibata et al., 2004). The pathological alterations in these lesions are identified by destruction of the gliosis and the axons, and diffuse demyelination, but the reasons leading to these changes have not been well identified (Seewann et al., 2009; Vellinga et al., 2009; Kezele et al., 2008).

In the normal conditions, microglia are highly ramified cells that display a quiescent phenotype. However, under pathological conditions, they are activated and are contributed to play a vital role to induce inflammation of the CNS (Kawabori and Yenari; Goldmann et al.; Miron et al.). Microglia are similar to other tissue macrophages, regulating immunity, inducing of cytokines and expression of matrix metalloproteinases (Zuiderwijk-Sick et al., 2007; Dijkstra et al., 2006; Jack et al., 2005).

MCP-1 is identified as one of the chemokines that is expressed in the central nervous system (Sato et al., 2009; Xu et al., 2009; Magge et al., 2009). Studies have demonstrated that up-regulation of MCP-1 in select central nervous system lesions, induced by hypoxia and inflammation (Sullivan and Zahr, 2008; Jiang et al., 2008; Kaminsky and Rogers, 2008). Up-regulation of MCP-1 induces microglia activation and promotes their migration to the lesions via interaction with its receptor, CCR2 (de Oliveira et al., 2009; Montecucco et al., 2008; Papadopoulou et al., 2008).

While the neuroinflammatory mechanisms play more predominant roles in the progression of WM lesions, less is known about the role of MCP-1 in WM lesions induced by chronic cerebral hypoperfusion. Therefore, in the present study, we evaluated the effect of MCP-1 on the WM lesions after chronic cerebral hypoperfusion.

## 2. Materials and methods

### 2.1. Animals

C57BL/6 mice (male, 8–10 weeks, 20–24 g) were obtained from the Animal Center of the Chongqing Medical University (Chongqing, China). Animals were housed in individual cages with free access to sterile acidified water and irradiated food in a specific pathogen-free facility at the Chongqing Medical University. Experiments were conducted in accordance with animal care guidelines approved by the Animal Ethics Committee of the Chongqing Medical University.

### 2.2. Recombinant adenovirus generation

The complementary DNA sequence of MCP-1 was obtained from GenBank. The potential target sequence for RNA interference (RNAi)

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were scanned with the small interfering RNA (siRNA) Target Finder and Design Tool available at the Ambion Website. The target sequence selected, 5′-GGAGCAGGCAUGGAAGUGUTT-3′ (sense),5′-ACA CUUCCG CCUGCUCCTT-3′ (antisense). The target sequence was subcloned into shuttle vector pDC315 and sequenced. The desired replication-deficient adenovirus containing the full-length cDNA of RNAi was generated by homologous recombination through co-transfection of plasmids pDC315-RNAi and pBHG1oXE1, 3Cre in HEK 293 cells using the DOTAP liposome reagent (Roche, Mannheim, Germany). After several rounds of plaque purification, the adenovirus was amplified and purified from cell lysates by banding twice in CsCl density gradients. Viral products were desalted and stored at −80 °C in PBS containing 10% glycerol (v/v). The infectious titer was determined by a standard plaque assay. In addition, the Ad-siRNA/LacZ which encodes LacZ shRNA was used as a negative control.

### 2.3. Intracerebroventricular injection

Guide cannulas were implanted stereotaxically (bregma 20.22 mm, lateral 11 mm, depth 22.5 mm) into the right lateral cerebral ventricle of the brain under halothane anesthesia (1.5% in $NO_2/O_2$, 70/30%), 5–7 d before bilateral common carotid artery stenosis (BCAS) surgery, to permit administration of 10 μl ($1 \times 10^9$ pfu) adenovirus intracerebro -ventricularly.

### 2.4. Bilateral common carotid artery stenosis (BCAS) surgery

Mice were subjected to BCAS, which was performed by applying the microcoils (Sawane Spring, Osaka, Japan) with an inner diameter of 0.18 mm to common carotid arteries (CCAs). Briefly, both CCAs were exposed and freed from their sheaths, through a midline cervical incision, after the mice were intraperitoneally anesthetized with sodium pentobarbital (50 mg/kg). Two 4 to 0 silk sutures were placed around the distal and proximal parts of the right CCA. Then, the artery was gently lifted by these sutures and placed between the loops of the microcoil just below the carotid bifurcation. The microcoil was twined by rotating it around the CCA. After 30 min, another microcoil of the same size was twined around the left CCA. The rectal temperature was maintained between 36.5 °C and 37.5 °C. Sham-operated mice underwent the same surgical procedure without using coils.

### 2.5. Klüver–Barrera staining

Fourteen days after BCAS, the animals were deeply anesthetized with pentobarbital and transcardially perfused with 0.9% saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). The brains were removed and postfixed for 12 h in 4% paraformaldehyde in 0.1 M PB (pH 7.4), and then stored in 30% sucrose in 0.1 M PB (pH 7.4). The corpus callosum was evaluated for WML by Klüver–Barrera staining. The myelin areas in three sections per animal and both sides of selected areas were stained with Luxol Fast Blue. The severity of WML was graded as 0 (normal), 1 (disarrangement of nerve fibers), 2 (formation of marked vacuoles), and 3 (disappearance of myelinated fibers).

### 2.6. Immunohistochemistry

The mice were anesthetized by intraperitoneal injection of chloral hydrate (350 mg/kg) and perfused through heart with 0.01 M phosphate-buffered saline (pH 7.6) and then with 4% paraformaldehyde. Brain tissues were removed and fixed in 4% paraformaldehyde for 3 d and then transferred in 0.1 M phosphate-buffered saline containing 30% sucrose for incubating at 4 °C overnight. The brain tissues were cryosectioned in the thickness of 30 μm for immunohistochemical staining. The sections were pretreated with 1% $H_2O_2$ for 10 min, blocked with 5% fetal calf serum in PBS for 30 min, and incubated with primary antibody at 4 °C overnight. The primary antibody used in this study included rabbit anti-CD11b polyclonal antibody (1:200, Santa Cruz). After washing, sections were incubated for 2 h with secondary anti-rabbit/goat IgG antibodies (1:300). The immunoreactivity was visualized by the avidin–biotin–peroxidase method (ABC) kit (Vector, Burlingame, CA, USA) combined with 0.05% 3,3′-diaminobenzidine (DAB) and 0.01% $H_2O_2$ for 5 min.

### 2.7. Western blot assay

Mice brain tissues were dissected and homogenized in T-PER buffer (Biosource Inc., USA) in the presence of protease inhibitors (Biosource Inc., USA). After homogenization, the lysates were centrifuged at 100,000 ×g, and the supernatants were saved for Western blotting Protein Chip Array. The samples of each supernatant and the final pellets were heat-blocked for 10 min in a loading buffer (125 mM Tris-HCl, 20% glycero1, 10% 2-mercaptethanol, 4% SDS, 0.02% bromophenol blue, pH 6.8) and then subjected to electrophoresis on a 10–20% Tris-glycine sodium dodecyl sulfate–polyacrylamide lamide gel (Sigma, USA). The samples were then electrically transferred to a transfer membrane (Biosynthesis co., China) and blocked for 1 h in phosphate-buffered saline (PBS) containing 10% skim milk and 0.1% Tween. Immunoblots were probed with specific antibodies: rabbit polyclonal TNF-α (1:500, Affiniti Research, Devon, United Kingdom), rabbit monoclonal IL-1β (1:1000, Lab Vision Corporation, CA, USA), and rabbit polyclonal IL-6 (1:1000, Lab Vision Corporation, CA, USA) were incubated at 4–8 °C overnight in a PBS buffer containing 5% bovine albumin. The membrane was rinsed with PBS buffer containing 0.1% Tween 20, incubated with HRP-labeled anti-rabbit IgG (1:5000, Biosynthesis co., China) for 3 h, and then stained with the detection reagents (Biosynthesis Co., China). An endogenous control protein, β-actin, was included in each Western blot. The optical densities of the specific bands were scanned and measured by image analysis software (HPIAS 2000, Tongji Qianping Company, Wuhan, China) and normalized to β-actin.

### 2.8. Real-time PCR

In brief, mice were sacrificed and hippocampi were then excised, immediately frozen in liquid nitrogen, and kept frozen at −80 °C. Total RNA was extracted using Trisol (Invitrogen, Carlsbad, CA) and reverse transcribed into cDNA using the reagents and protocol of the PrimeScript RT reagent kit (TaKaRa BIO INC., Kyoto, Japan). The RT reaction was performed with a GeneAmp PCR System 2700 (PerkinElmer, Boston, MA). The primers for MCP-1 were sense 5′-AGCTTTCATTTCGA AGTCTTTG-3′ and anti-sense 5′-TAGA TTC GG TTTAATTGGCCC-3′; β-actin forward: 5′-GG CATCGTGATG GAC TC CG-3′ and anti-sense 5′-GC TGGA AGG TGG ACAGCGA-3′. For quantification of the mRNA expression, real-time PCR was performed with SYBR Premix Ex Taq II reagent (TaKaRa BIO INC.) using an ABI PRISM 7900 system (Applied Biosystems, Lincoln, CA). Relative copy numbers were obtained from the standard curve values and were normalized to the values obtained for the housekeeping gene, Actb. Expression levels of each mRNA were normalized to the percentage of the intact controls, taken as 100%. All amplification products were confirmed by the presence of a single peak in the dissociation curve after the PCR reaction to check the specificity of the amplification.

### 2.9. Water maze test

The Morris water maze test was used to evaluate the spatial memory in rodents. In a 200-cm-diameter circular pool filled with 24-cm-deep water at a temperature of 22 ± 1 °C, a circular acrylic platform (15 cm in diameter) was prepared, the top surface of which was 2 cm below the water. A submerged platform was then placed in one of the pool quadrants. Rats were released facing the wall, and the time taken to escape to the platform was recorded as the escape latency. The time and path to reach the platform were recorded by a video-computerized

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