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Interleukin 8 (CXCL8)-CXC chemokine receptor 2 (CXCR2) axis contributes to MiR-4437-associated recruitment of granulocytes and natural killer cells in ischemic stroke

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ABSTRACT

Granulocytes and natural killer (NK) cells have been linked to brain injury in ischemic stroke. However, their recruitment from peripheral leucocytes in stroke patients is not well understood. Here, the expression of the interleukin 8 (CXCL8) in plasma, and CXC chemokine receptor 2 (CXCR2) in peripheral leucocytes of patients with ischemic stroke were evaluated. Based on the results, CXCR2 expression positively correlated with granulocytes and NK cells, which were in turn attracted by CXCL8. The results also indicated that CXCR2 was a direct target of microRNA (miR)-4437, a negative regulator of CXCR2, which was downregulated in peripheral leucocytes from patients with ischemic stroke. Furthermore, serum CXCL8 levels were associated with the infarct volume and functional outcomes in patients with ischemic stroke. The results of the receiver operating characteristic curve analysis with an optimal cut-off value of 34 pg/mL indicated serum CXCL8 levels could be a prognostic indicator for ischemic stroke. In conclusion, these data highlighted the involvement of the CXCL8-CXCR2 chemotactic axis in the recruitment of granulocytes and NK cells in ischemic stroke. Furthermore, miR-4437 was suggested as a novel target for treating ischemic stroke, while the serum CXCL8 level could be a prognostic factor for ischemic stroke.

1. Introduction

Accumulating evidence has indicated that the immune system and inflammation play a leading role in ischemic stroke, particularly in brain tissue damage, progression of ischemic lesions, and tissue repair (Burrows et al., 2016; Ma et al., 2015; Picascia et al., 2015). Typically following ischemic stroke there is a rapid infiltration of granulocytes, particularly neutrophils, which can result in blood-brain barrier disruption, cerebral edema, and brain injury (Jickling et al., 2015). In addition to granulocytes, natural killer (NK) cells, which are key members of the innate immune system, accumulate in the ischemic hemisphere (Gan et al., 2014; Zhang et al., 2014). The NK cells determine the size of the brain infarct (Gan et al., 2014) and are responsible for ischemia-related neuronal death by secreting interferon

Abbreviations: NK, natural killer; PBMCs, peripheral blood mononuclear cells; CXCL8, interleukin-8; CXCR2, CXC chemokine receptor 2; IFN, interferon; miR, microRNA; AHA/ASA, American Heart Association/American Stroke Association; DWI, diffusion-weighted imaging; mRS, modified Rankin Scale; DMEM, Dulbecco's modified eagle medium; FBS, fetal bovine serum; NC, negative control; RT-PCR, real-time reverse transcription polymerase; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate buffered saline; PerCP, peridinin-chlorophyll-protein; CD, cluster of differentiation; PE-CY7, phycoerythrin-cyanin 7; APC, allophycocyanin; FITC, fluorescein isothiocyanate; FACS, Fluorescence-Activated Cell Sorting; GEO, Gene Expression Omnibus; SD, standard deviation; ROC, receiver operating characteristic; WT, wildtype; AUC, area under the curve; TNF, tumor necrosis factor

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(IFN)-γ (Gan et al., 2014; Zhang et al., 2014). On the other hand, chemokines are a class of pro-inflammatory cytokines that have the ability to attract and activate leukocytes. In the highly inflammatory sites of acute ischemic stroke, chemokines are mainly generated by microglial cells and infiltrating immune cells, resulting in an exacerbated inflammatory cascade and brain injury (Lee et al., 2015; Remus et al., 2015).

The CXC chemokine receptor 2 (CXCR2) is a regulator of neutrophil homeostasis (Veenstra and Ransohoff, 2012). In experimental mice with ischemic stroke, it has been demonstrated that a vast amount of CXCR2+ granulocytes are activated and released from the bone marrow (Denes et al., 2011). Furthermore, CXCR2 blockade mitigated the neurological deficits and reduced the infarct volume (Herz et al., 2015; Sousa et al., 2013). Thus, CXCR2-positive granulocytes play an important role in ischemic brain injury. However, no study to date has evaluated the expression of CXCR2 in patients with stroke. Additionally, in ischemic stroke the expression of interleukin-8 (CXCL8), one of the ligands of CXCR2, is controversial. In particular, several previous studies have indicated no change in CXCL8 levels in patients with stroke (Montaner et al., 2003; Pedersen et al., 2004), while others demonstrated increased CXCL8 levels (Al-Bahrani et al., 2007; Grau et al., 2001a, b). Furthermore, the involvement of the CXCL8-CXCR2 chemotactic axis in the recruitment of immune cells in ischemic stroke is not well understood.

This study aimed to investigate the effect and underlying mechanism of the CXCL8-CXCR2 chemotactic axis in ischemic stroke.

2. Materials and methods

2.1. Patients

A total of 140 patients experiencing the first week of their first-ever acute ischemic stroke were recruited at the First Affiliated Hospital of Zhengzhou University from 25 June 2015 to 20 June 2018. All enrolled patients signed a written informed consent, and all experimental protocols were approved by the Ethics Committees of the First Affiliated Hospital of Zhengzhou University. Patients were excluded if they had infections, autoimmune diseases, cancers, or other conditions that could impact immune system homeostasis. During this study, the diagnosis and treatment of ischemic stroke were carried out under the guidelines of the American Heart Association/American Stroke Association (AHA/ASA) (Jauch et al., 2013). Table 1 summarizes the basic characteristics of the enrolled patients. As controls, a total of 70 age- and sex-matched healthy donors were also recruited. The infarct volume was measured by diffusion-weighted imaging (DWI) according to the validated ABC/2 method (Sims et al., 2009). Most of the patients completed a 90-day clinical follow-up. The functional outcome was assessed using the modified Rankin Scale (mRS) with the CXCL8 levels being concealed. The mRS is typically used to evaluate the degree of disability or dependence in the daily activities of patients following stroke. The favorable functional outcome was defined by an mRS score of 0–2 points (0, no symptoms; 1, no significant disability; and 2, slight disability), while the unfavorable outcome was defined by an mRS score of 3–6 points (3, moderate disability; 4, moderately severe disability; 5, severe disability; and 6, deceased). In this study, we analyzed the relationship between ischemic stroke and peripheral leucocytes. According to cellular morphology, leucocytes can be divided into two groups: peripheral blood mononuclear cells (PBMCs) and polymorphonuclear cells. Polymorphonuclear cells were isolated by single-step centrifugation of whole blood using Polymorphprep (Axis-Shield) as described previously (Thomas et al., 2015). Furthermore, PBMCs were collected using Ficoll-Hypaque density gradient centrifugation (Beijing Chemical Reagent Company, China) as described previously (Liu et al., 2015). Finally, we put these two groups of cells together for further testing.

Table 1
Patient Characteristics.

Variables	Study patients (n = 140)
Age, years (mean ± SD)	58.47 ± 10.91
Male sex, % (n)	69.29 (97)
Han race, % (n)	100 (140)
Time to enrollment, days (mean ± SD)	3.24 ± 2.57
DWI volume, mL (mean ± SD)	17.43 ± 9.05
Hypertension, % (n)	77.86 (109)
Diabetes, % (n)	28.57 (40)
Atrial fibrillation, % (n)	3.57 (5)
Hypercholesterolemia, % (n)	13.57 (19)
Smoke, % (n)	33.57 (47)
Drink, % (n)	26.42 (37)
Triglyceride (mmol/L)	1.50 ± 0.95
Cholesterol (mmol/L)	4.26 ± 1.00
Homocysteine (μmol/L)	15.96 ± 8.89
Baseline NIHSS Score (mean ± SD)	4.19 ± 3.56

DWI, diffusion weighted imaging; NIHSS, National Institutes of Health Stroke Scale; SD, standard deviation.

2.2. Cell culture and transfection

293 T cells were cultured at 37 °C in a 5% CO2-humidified incubator with Dulbecco's Modified Eagle Medium (DMEM) high-glucose supplemented with 10% fetal bovine serum (FBS). The microRNA (miR)-4437 mimic and negative control (NC) were chemically synthesized (Shanghai GenePharma Company, Shanghai, China) and had the following sequences: miR-4437 mimic, 5′-UGGGCUCAGGGUACAAAG GUU-3′ and 5′−CCUUUGUACCCUGAGCCCAUU-3′; NC, 5′-UUCUCCG AACGUGUCACGUTT-3′ and 5′-ACGUGACACGUUCGGAGAATT-3′. Cells were seeded onto 24-well plates at a density of 4 × 104 cells/well and co-transfected with oligonucleotides (100 nM, miR-4437 mimics or NC) and plasmids (500 ng, pmirGLO-CXCR2-3′UTR-WT or pmirGLO-CXCR2-3′UTR-MUT). All procedures were performed using the Lipofectamine 3000 reagent (Invitrogen, Carlsbad, USA) following the manufacturer's instructions. Forty-eight hours after transfection, cells were harvested for the luciferase activity assay.

2.3. Plasmid construction and luciferase activity assay

The 60-bp sequence of the CXCR2 3′UTR, containing the predicted miR-4437 binding sites, and its mutant, were synthesized as primers by Sangon Biotech (China, Shanghai). Subsequently, sense and antisense oligonucleotides were mixed with 5X annealing buffer (Beyotime, China). The mixture was boiled for 10 min and then cooled slowly to ambient temperature. The double-stranded oligonucleotides were digested with SacI and Xhol. The resulting oligonucleotides were subcloned into the SacI and Xhol sites of the pmirGLO vector. Luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega) on a Cetro XS3 LB 960 microplate luminometer (Berthold, Germany), according to the manufacturer's protocol.

2.4. RNA extraction and real-time reverse transcription polymerase chain reaction (RT-PCR)

Total RNA of PBMCs and polymorphonuclear cells were extracted using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). To determine miRNA expression, the RNA was subsequently reverse-transcribed using the miScriptII RT Kit (Qiagen) according to the manufacturer's instructions. The expression levels of miR-1305 and miR-4437 were quantified with SYBR Green Master (Roche) using a miRNA-specific forward primer and a universal poly (T) adaptor reverse primer. The U6 small nuclear RNA served as the internal reference. Real-time PCR was performed on an Agilent Mx3005 P Real-time PCR system using FS Universal.