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Iron released from reactive microglia by noggin improves myelin repair in the ischemic brain

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ABSTRACT

We previously reported that the bone morphogenetic protein (BMP) antagonist, noggin, improved the repair process with an increase in the reactive microglia/macrophage population in the ischemic brain. Since BMP plays a role in intracellular iron homeostasis via the hepcidin/ferroportin axis, and iron is required for myelination, this study was aimed to determine whether noggin affected iron status and remyelination in the brain following ischemic stroke. We further examined the effect of blocking the BMP/hepcidin pathway on reactive microglia (BV2) and myelination of oligodendroglial cells (MO3.13) to define the link between microglial iron status and myelin formation. Following the noggin infusion into the ischemic brain of mice, the induction of hepcidin and ferritin protein levels decreased, and the number of myelinated axons and myelin thickness increased at 8 weeks after ischemic stroke. Noggin repressed the increase in hepcidin and ferritin levels in BV2 exposed to lipopolysaccharide (LPS) and oxygen/glucose deprivation and reperfusion (OGD/R). When MO3.13 were exposed to the conditioned media from noggin-treated BV2 (noggin CM) during reperfusion, OGD/R-induced MO3.13 cell death was reduced. Under normal conditions, noggin CM induced myelin production with an increase in ferritin levels in MO3.13, which was reversed by the iron chelator, deferoxamine. These results indicated that noggin altered the iron status in reactive microglia from the iron-storing to the iron-releasing phenotype, which contributed to myelin synthesis by providing iron. We suggest that the BMP/hepcidin pathway can be a target for the regulation of the iron status in microglia to enhance remyelination in the ischemic brain.

1. Introduction

Ischemic stroke, which accounts for the majority of all cases of stroke, is a major cause of disability in adults (Mozaffarian et al., 2016). Except for thrombolysis during the acute phase (Hacke et al., 2008), there is currently no specific medication to improve neurological recovery during the subacute or chronic phase in patients who survive ischemic stroke. Since spontaneous recovery can be observed weeks later, studies to define the underlying mechanisms of endogenous repair processes such as neurogenesis, neural plasticity, angiogenesis, and remyelination are warranted, so that neurorestorative therapies for long-term recovery following ischemic stroke can be developed (Chen et al., 2014).

It has been shown that transcription of myelin basic protein (MBP), a major protein of the myelin sheath, was increased in the peri-infarct area at 7 days after transient ischemic stroke (Gregersen et al., 2001). Furthermore, the number of mature oligodendrocytes, which are myelin-forming glial cells in the brain, was increased in the ischemic striatum during the chronic stage (Zhang et al., 2011). These studies indicate that myelin formation from oligodendrocytes is induced by ischemia and is involved in the remyelination of damaged axons during the recovery phase. Oligodendrocytes are the predominant iron-containing cells in the brain; they contain iron in the form of ferritin (Connor and Menzies, 1996). Iron is an essential cofactor for enzymes involved in myelin

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cholesterol and fatty acid synthesis, and therefore the adequate import of extracellular iron is required for the function and activity of oligodendrocytes (Todorich et al., 2009). Microglia are suggested to be a source of iron for oligodendrocytes, by releasing ferritin (Zhang et al., 2006).

The cellular iron level in immune cells, including macrophages and microglia, is controlled by hepcidin and ferroportin (Ward et al., 2011). Hepcidin is mainly produced in the liver, and its transcription is regulated by the bone morphogenetic protein (BMP)/Sma and Mad Related Family (Smad) signaling pathway, and the interleukin-6 (IL-6)/Janus kinase 1/2-signal transducer and activator of transcription 3 (STAT3) signaling pathways during inflammation (Schmidt, 2015). The induction of hepcidin results in the degradation of the only known iron exporter ferroportin, subsequently increasing the level of intracellular iron (Li et al., 2016; Urrutia et al., 2013). Recent studies have demonstrated that hepcidin is produced in microglia/macrophages by pro-inflammatory stimuli such as IL-6, tumor necrosis factor α (TNF-α), or lipopolysaccharide (LPS), and have suggested its paracrine and autocrine effects (Li et al., 2016; Urrutia et al., 2013; Wu et al., 2012). In addition, it was reported that hepcidin is induced by exogenous administration of BMP4 or BMP6 in LPS-stimulated macrophages, indicating the role of BMP signaling in hepcidin regulation in immune cells (Wu et al., 2012). Therefore, it can be postulated that locally produced BMPs affect the intracellular iron levels of microglia and macrophages, and also influences iron availability in the tissue.

We previously reported that levels of BMPs and an endogenous BMP antagonist, noggin, were differentially induced in the brain at 1 week to 4 weeks after an ischemic stroke (Shin et al., 2012). In a subsequent study, we demonstrated that intracerebroventricular infusion of noggin after an ischemic insult improved the repair processes and was accompanied by the increase of reactive microglia/macrophages in the brain of mice (Shin et al., 2014). In the present study, we examined long-term functional outcomes, myelin production, and the iron status in the brain treated with noggin at 8 weeks after ischemic stroke. In addition, changes in the iron status of reactive microglia by noggin treatment and their effects on myelin production in oligodendrocytes *in vitro*; the aim was to define the role of blocking the BMP/hepdicin pathway in reactive microglia/macrophages and remyelination in the brain during the recovery phase after ischemic stroke.

2. Material and methods

2.1. Experimental animals

The current study was conducted using male C57BL/6 mice, aged 10–11 weeks (Orient Bio Inc., Seongnam, Republic of Korea). All procedures were approved by the Institutional Animal Care and Use Committee at the Medical School of Ewha Womans University, and were conducted in according with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978). The number of animals used was minimized to reduce animal suffering.

2.2. Transient middle cerebral artery occlusion

Transient middle cerebral artery occlusion (MCAO) was induced as previously described (Shin et al., 2014). Briefly, a 6-0 silicon-coated black monofilament surgical suture (Doccol Cooperation, Redlands, CA, USA) was wedged into the circle of Willis to obstruct the origin of the middle cerebral artery via the internal carotid artery of mice anesthetized with isoflurane. The filament was left in place for 30 min and then withdrawn to re-establish cerebral blood flow (CBF), which was monitored by a laser-Doppler flowmeter (Periflux System 5010, Perimed, Sweden). Only animals that exhibited a greater than 85% reduction in CBF during MCAO, which recovered by more than 80% after 10 min of reperfusion, were included. Rectal temperature was maintained at 37.0 ± 0.5 °C, with a thermostatically controlled heating pad, during surgery and recovery until mice regained consciousness.

2.3. Intraventricular infusion

Recombinant human noggin (R&D Systems, Minneapolis, MN, USA) dissolved in artificial cerebrospinal fluid (aCSF; R&D Systems), or aCSF alone, was delivered to the ipsilateral lateral ventricle using an ALZET osmotic pumps (DURECT Corporation, Cupertino, CA, USA). A cannula (ALZET Brain infusion kit 3, DURECT Corporation) connected to the osmotic pumps was implanted 0.5 mm anterior to bregma, 1 mm lateral from the midline, and 3 mm below the surface of the skull. Mice received 1 μg of protein per day for 2 weeks, starting 2 weeks after the MCAO, as previously reported (Shin et al., 2014). Thereafter, infusion pumps were explanted 4 weeks after the MCAO (Fig. 1A). Animals were assigned to each treatment randomly using computerized random number generators, and the MCAO, measurements of brain volumes, and analysis of behavioral outcomes by researchers who were blinded to drug assignment.

2.4. Behavioral tests

To examine the physiological significance of noggin treatment after stroke, three behavioral tests were performed in mice. Neurological scores and performance in the wire suspension test and grips strength test were examined prior to, and weekly after, the MCAO. The body weight was also measured weekly prior to, and for 8 weeks after, the MCAO. A 5-point graded scoring system for neurological scores and the wire suspension test was used (Shin et al., 2015). Forelimb strength (in newtons, N) was measured with a grip strength tester (BIO-GS3, Bioseb, Vitrolles Cedex, France) to detect contralateral paw weakness. After both forelimbs of the mouse were loosened by pulling the tail, the maximal force was recorded. Each mouse was subjected to five trials of each test, the mean values (N) were calculated and normalized to body weight, and the percent changes from the baseline values, which were measured prior to the MCAO, were analyzed.

2.5. Measurement of hemispheric brain volume

Mice were euthanized 8 weeks after the MCAO. The brains were removed, frozen, and sectioned (30 μm thick) using a cryostat. Brain sections were collected serially at 600-μm intervals and stained with cresyl violet. Each hemispheric volume was measured with an image analyzer (Axiovision LE 4.1, Carl Zeiss, Jena, Germany), and the ischemic brain atrophy was expressed as a percentage of the ipsilateral hemispheric volume to the contralateral hemispheric volume.

2.6. Transmission electron microscopic analysis

Small blocks from the ipsilateral cortex were processed for electron microscopy, and embedded in Epon resin, as previously described (Shin et al., 2015). Areas of interest were selected from 1-μm-thick semi-thin sections cut with an ultra-microtome (Reichert-Jung, Leica, Wetzlar, Germany) and stained with toluidine blue. Ultrathin (60–70 nm) sections were cut, stained with 1% uranyl acetate and 1% lead citrate (Ted Pella, Inc., Redding, CA, USA), and examined with an electron microscope (Hitach 7650; Hitach, Tokyo, Japan). Images were obtained using a Morada camera (Soft