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MMP-3 secreted from endothelial cells of blood vessels after spinal cord injury activates microglia, leading to oligodendrocyte cell death

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ABSTRACT

The activation of microglia after spinal cord injury (SCI) contributes to secondary damage by producing pro-inflammatory cytokines and mediators, leading to cell death of oligodendrocytes and neurons. Here, we show that matrix metalloprotease-3 (MMP-3) produced and secreted in the endothelial cells of blood vessels after SCI mediates microglial activation. MMP-3 was produced and secreted in bEnd.3 cells, a mouse brain-derived endothelial cell line, by oxygen–glucose deprivation/reoxygenation (OGD/RO). OGD/RO-induced MMP-3 expression and activity was also significantly inhibited by ghrelin, which was dependent on the ghrelin receptor GHS-R1a. Furthermore, the secreted MMP-3 from OGD/RO-induced bEnd.3 cells activated BV-2 cells, a murine microglial cell line. We also found that microglial activation after SCI was attenuated in MMP-3 knockout (KO) mice compared with wild type (WT) mice. Both p38 mitogen-activated protein kinase (MAPK) activation and pro-nerve growth factor (proNGF) production were more inhibited in MMP-3 KO than WT mice at 5 d after injury. When WT mice were treated with *Mmp-3* siRNA after injury, MMP-3 activity, microglial activation, p38MAPK activation and proNGF expression were significantly inhibited. Ghrelin treatment also significantly inhibited MMP-3 expression and activation after SCI, which was dependent on GHS-R1a. Finally, RhoA activation and oligodendrocyte cell death after injury were attenuated by *Mmp-3* siRNA or ghrelin treatment compared with vehicle control. Thus, our study indicates that MMP-3 produced in blood vessel endothelial cells after SCI serves as an endogenous molecule for microglial activation followed by p38MAPK activation and proNGF production, and further indicates that the protective effect of ghrelin on oligodendrocytes cell death may be at least partly mediated by the inhibition of MMP-3-induced microglial activation after SCI.

Introduction

Oligodendrocytes, a glial cell that myelinates axons in the central nervous system (CNS), undergo delayed apoptotic cell death in degenerating white matter (WM) tracts distant from the injury site for weeks after spinal cord injury (SCI) (Springer et al., 1999; Casha et al., 2001a). Oligodendrocyte cell death well characterized temporally and spatially after SCI contributes to the chronic demyelination of spared axon through Wallerian degeneration and exacerbates the extent of injury, eventually leading to a permanent deficit of motor function (Beattie et al., 2000; Warden et al., 2001).

Inflammation and oxidative stress are known to be major factors that exacerbate apoptotic cell death of oligodendrocytes after SCI (Stirling et al., 2004; Yune et al., 2007; Yu and Fehlings, 2011; Moon et al., 2012; Valluru et al., 2012). Particularly, microglia are activated within minutes to hours after SCI and transformed into macrophages, which contribute to damage by releasing pro-inflammatory cytokines and reactive oxygen species (ROS), leading to tissue destruction, enlargement of the lesion and the apoptotic cell death of oligodendrocytes and neurons (Popovich et al., 1997, 2002; Taoka et al., 1997; Chatzipanteli et al., 2002; Stirling et al., 2004; Gris et al., 2004; Yune et al., 2007). It was also reported that p38 mitogen-activated protein kinase (p38MAPK) is activated in microglia and thereby mediates the production of pro-nerve growth factor (proNGF), which is involved in the apoptotic cell death of oligodendrocytes after SCI (Yune et al., 2007). Therefore, to develop effective therapeutic interventions for blocking oligodendrocyte cell death after SCI, the inhibition of inflammation and oxidative stress by attenuating microglial activation should be considered.

Abbreviations: CNS, central nervous system; COX-2, cyclooxygenase-2; iNOS, inducible nitric oxide synthase; IL, interleukin; KO, knockout; LPS, lipopolysaccharide; MMPs , matrix metalloproteases; NO, nitric oxide; OGD/RO, oxygen glucose deprivation/reoxygenation; p38MAPK, p38 mitogen-activated protein kinase; proNGF, pro-nerve growth factor; ROS, reactive oxygen species; SCI, spinal cord injury; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphatebiotin nick end labeling; TNF-α, tumor necrosis factor α; WM, white matter; WT, wild type.

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Although the role of activated microglia in neurodegeneration has been well documented (Cunningham, 2013), the endogenous molecules that trigger microglial activation remain to be identified. Matrix metalloproteases (MMPs) are known to degrade the extracellular matrix and other extracellular proteins (Sternlicht et al., 1999; Sternlicht and Werb, 2001) and are essential for remodeling the extracellular matrix and wound healing (Werb, 1997). Additionally, MMP-3 is known to play an important role in the remodeling of extracellular macromolecules and has been linked to various pathological conditions in the CNS, such as ischemia, multiple sclerosis, Alzheimer's disease, and Parkinson's disease (Maeda and Sobel, 1996; onso de and Egido, 2006; Kim et al., 2007; Walker and Rosenberg, 2009; Reitz et al., 2010; Lee et al., 2014a). In particular, the report by Kim et al. (2005) showed that MMP-3 released from apoptotic neurons in the early stage of cell death is one of the molecules that activates microglia. Additionally, MMP-3 released from stressed dopamine neurons has been known to induce microglial activation and superoxide generation, eventually leading to the degeneration of nigrostriatal dopamine neurons (Kim et al., 2007). Recently, we also reported that MMP-3 is expressed in the endothelial cells of blood vessels after SCI (Lee et al., 2014a), but the role of MMP-3 in microglial activation after SCI has not been examined.

Here, we report that MMP-3 produced and secreted in the endothelial cells of blood vessels after SCI induces microglial activation followed by p38MAPK activation and proNGF production, thereby mediates oligodendrocytes cell death through RhoA activation and c-Jun phosphorylation. In addition, ghrelin treatment attenuates microglial activation by inhibiting MMP-3 expression, which is dependent on the ghrelin receptor (GHS-R1a) and thereby alleviates oligodendrocyte cell death after SCI.

Materials and methods

Spinal cord injury

We used adult (13–16 weeks old, 28–30 g) male wild type (WT, n = 149) mice and MMP-3 knockout (KO, n = 11) mice generously donated by Dr. Tong H. Joh (Cornell University, USA) in this study. The genetic background of MMP-3 KO mice was C57BL/6 and the same strain from Samtako (Osan, Korea) was used as a WT. Mice were subjected to moderated contusion injury (50 kdyn force per 500 to 600 μm displacement) using the Infinite Horizons (IH) impactor (Infinite Horizons Inc., Lexington, KY) as described previously (Lee et al., 2012b). Surgical interventions and postoperative animal care were performed in accordance with the Guidelines and Policies for Rodent Survival Surgery provided by the Animal Care Committee of the Kyung Hee University.

Drug treatment

Acylated ghrelin (Peptides International, Louisville, KT) was dissolved in sterile PBS and administered by intraperitoneal (i.p.) injection. Mice were given with ghrelin (80 μg/kg) immediately after SCI and then received the same dose of ghrelin every 6 h for 1 d. To determine whether ghrelin acts *via* GHS-R1a, an antagonist of GHS-R1a, [D-Lys3]-GHRP-6 (Bachem, Torrance, CA) was dissolved in sterile PBS and [D-Lys3]-GHRP-6 (3 mg/kg, i.p.) was given at 30 min before every ghrelin injection (immediately, every 6 h for 1 d after injury). Vehicle control groups received equivolumetric i.p. injection of PBS at the corresponding time points. For the sham-operated controls, mice underwent a T9–T10 laminectomy without contusion injury, and received no pharmacological treatment. Mouse *Mmp-3* siRNA and control non-targeting siRNA (control siRNA) were purchased from Dharmacon RNA Technologies (Lafayette, CO). Mmp-3 targeted siRNA (SMART pool) was a mixture of 4 siRNAs formulated to knockdown Mmp-3 gene effectively. The control siRNA was a mixture of siRNAs with scrambled sequences. siRNAs were dissolved in sterile PBS and injected bilaterally (2 μl/site, final 0.05 nmol per cord) into the spinal cord in WT mice at 30 min after SCI as previously described (Lee et al., 2014a).

Tissue preparation

At 1, 3, or 5 d after SCI, mice were anesthetized with chloral hydrate (500 mg/kg) and perfused *via* cardiac puncture initially with 0.1 M PBS, pH 7.4 and subsequently with 4% paraformaldehyde in 0.1 M PBS. A 20-mm section of the spinal cord, centered at the lesion site, was dissected out, post-fixed by immersion in the same fixative overnight and placed in 30% sucrose in 0.1 M PBS, pH 7.4. The segment was embedded in OCT for frozen sections, and longitudinal or transverse sections were then cut at 10 or 16 μm. For molecular works, animals were perfused with 0.1 M PBS and segments of spinal cord (8 mm) including the lesion site were isolated and frozen at −80 °C until use.

bEnd.3 cell culture and oxygen glucose deprivation/reoxygenation (OGD/RO)

A mouse brain endothelial cell line, bEnd.3 (ATCC, Manassas, VA) was cultured as previously described (Lee et al., 2012b). Prior to each experiment, cells were seeded onto 6-well (5×10^5 cells/well) plate. On the next day, the cells were treated with ghrelin (10 nM) in the presence or absence of [D-Lys3]-GHRP-6 (100 μM). [D-Lys3]-GHRP-6 was treated for 30 min before ghrelin treatment. For vehicle-control, PBS without ghrelin or [D-Lys3]-GHRP-6 was used. To achieve OGD/RO, culture medium was replaced with glucose-free DMEM (Sigma, St. Louis, MO) and then placed in a Hypoxic Workstation (Daiki Sciences Co. Ltd. by Ruskinn Technology, Bridgend Mid Glamorgan, UK) at 37 °C in a humidified atmosphere containing a gas mixture of 0.1% O_2, 5% CO_2, and 94.9% N_2. After 6 h of OGD, cells were placed under normoxic conditions and the media was quickly replaced with 25 mM glucose-containing DMEM. Control cells were cultured in DMEM with 25 mM glucose under normoxia.

BV-2 cell culture and drug treatment

An immortalized murine BV-2 cell line (Blasi et al., 1990) was cultured in DMEM supplemented with 5% FBS and 100 units/ml penicillin and 100 μg/ml streptomycin at 37 °C in a humidified incubator under 5% CO_2 as previously described (Yune et al., 2007). Prior to each experiment, cells (5×10^5 cells/well) were plated onto 6-well plate. On the next day, BV-2 culture media were replaced with conditioned media (200 μl, 20% of media volume) obtained from bEnd.3 cells treated with OGD or OGD plus *Mmp-3* siRNA. Control cultures were treated with lipopolysaccharide (LPS, 100 ng/ml; *Escherichia coli* 0111:B4; Sigma).

Cell transfection

The siRNA library contained Smart-pool libraries (Thermo Fisher Scientific) of 4 siRNAs duplexes per gene. For siRNA transfection, cells were plated onto 6-well (5×10^5 cells/well) plate in complete medium. At 30 min before OGD, culture media was changed with glucose free DMEM and cells were transfected with siRNA at the final concentration of 33 nM using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction.

Assay for nitric oxide production

The production of nitric oxide (NO) was measured as described previously (Yune et al., 2009). Briefly, 100 μl of culture medium was allowed to react with 100 μl of Griess reagent (Sigma). The optical density was read at 540 nm in a microplate reader (Molecular Devices, Sunnyvale, CA) after 15 min (n = 3 wells/group). Nitrite concentrations were calculated from a standard curve derived from the reaction of sodium nitrite in fresh media.