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Research Paper

Antagonizing bone morphogenetic protein 4 attenuates disease progression in a rat model of amyotrophic lateral sclerosis

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ABSTRACT

Amyotrophic lateral sclerosis (ALS) is an adult-onset, fatal neurodegenerative syndrome characterized by the systemic loss of motor neurons with prominent astrocytosis and microgliosis in the spinal cord and brain. Astrocytes play an essential role in maintaining extracellular microenvironments that surround motor neurons, and are activated by various insults. Growing evidence points to a non-cell autonomous neurotoxicity caused by chronic and sustained astrocytic activation in patients with neurodegenerative diseases, including ALS. However, the mechanisms that underlie the harmful effects of astrocytosis in patients with ALS remain unresolved. We focused on bone morphogenetic proteins as a major soluble factor that promotes astrocytogenesis and its activation in the adult spinal cord. In a transgenic rat model with ALS-linked mutant Cu/Zn superoxide dismutase gene, BMP4 was progressively up-regulated in reactive astrocytes of the spinal ventral horns, whereas the BMP-antagonist noggin was decreased in association with neuronal degeneration. Continuous intrathecal noggin supplementation after disease onset significantly ameliorated motor dysfunction symptoms, neurogenic muscle atrophy, and extended survival of symptomatic ALS model rats, despite lack of deterrence against neuronal death itself. The exogenous noggin inhibited astrocytic hypertrophy, astrocytogenesis, and neuroinflammation by inactivating both Smad1/5/8 and p38 mitogen-activated protein kinase pathways. Moreover, intrathecal infusion of a *Bmp4*-targeted antisense oligonucleotides and provided selective *Bmp4* knockdown in vivo, which suppressed astrocyte and microglia activation, reproducing the aforementioned results by noggin treatment. Collectively, we clarified the involvement of BMP4 in the processes of excessive gliosis that exacerbate the disease progression of the ALS model rats. Our study demonstrated that BMP4, with its downstream signaling, might be a novel therapeutic target for disease-modifying therapies in ALS.

1. Introduction

Amyotrophic lateral sclerosis (ALS) is an adult-onset, fatal neurodegenerative syndrome where patients develop progressive muscle atrophy and weakness, ultimately leading to respiratory failure, generally within a few years of onset (Tandan and Bradley, 1985). To date, there are no available curative therapies for improving the symptoms or halting the devastating effects of motor neuron disease. The pathological hallmark in ALS is prominent and systemic loss of motor neurons in the central nervous system (CNS). This process involves marked activation of astrocytes and microglia, similar to other neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease (Maragakis and Rothstein, 2006).

Astrocytes are the major non-neuronal cells in the CNS and have a pivotal role in maintaining homeostasis of the extracellular microenvironment (Burda and Sofroniew, 2014). Under various pathological conditions, by both acute and chronic insults to the CNS, astrocytes develop enlarged cell bodies and processes, and strongly express intermediate filaments such as glial fibrillary acidic protein (GFAP), nestin, and vimentin (Ben Haim et al., 2015). When acute CNS injury occurs, perilesional astrocytes form neuroprotective glial scars, which prevent the injury from spreading to the intact regions. However, within the context of neurodegenerative conditions, chronically activated astrocytes have adverse neuronal effects. Growing evidence

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suggests that, in patients with ALS and ALS animal models, the reactive astrocytes lose their homeostatic functions secondary to glutamate uptake deficiencies, while expressing high levels of pro-inflammatory chemokines and cytokines (Yamanaka and Komine, 2018). Collectively, regulating the pathological activation of astrocytes may suppress disease progression in patients with ALS.

Resident CNS astrocytes have the ability to proliferate, in contrast to terminally differentiated neurons. In addition, throughout the mammalian spinal cord and brain, tissue-specific neural stem/progenitor cells, along with ventricular neuraxis and glial-restricted precursors dispersed throughout the parenchyma, are believed to exist, certainly in adulthood (Horner et al., 2000). Nearly any insult is likely to activate their proliferation and differentiation, producing astrocytes in vivo (Takahashi et al., 2003) (Lepore et al., 2008). Although mechanisms underlying insult-induced astrocytosis in the adult CNS are not fully elucidated, recent progress in developmental and stem cell biology has revealed a variety of extracellular molecules that promote astrocytogenesis and their subsequent maturation.

Bone morphogenetic proteins (BMPs) are glycoproteins that belong to the transforming growth factor beta (TGFβ) superfamily. These proteins are indispensable for neural development (Katagiri and Watabe, 2016), as well as for bone and cartilage formation. Among the BMP families, BMP2 and 4 promote astrocytic differentiation from neural stem cells, while inhibiting both neuronal and oligodendrocytic differentiation. In the adult mammalian CNS, BMP2, 4, noggin, and their receptors are expressed (Miyagi et al., 2012) and are involved in pooling neural stem cells by modulating their proliferation and differentiation (Katagiri and Watabe, 2016). BMP2, 4, 6 and 7 are up-regulated under neuropathological conditions such as spinal cord injury (Setoguchi et al., 2004) (Cui et al., 2015) and Alzheimer's disease (Tang et al., 2009). These observations suggest that BMPs play unresolved, but essential, roles in adult astrocytosis.

Considering the diverse cellular sources that facilitate astrocytic proliferation and differentiation, we focused on BMPs as potential endogenous regulators of astrocytosis. By investigating their temporal and spatial expressions in the adult rat spinal cord under an ALS-like neurodegenerative condition, we attempted to clarify possible relationships between BMP signaling and the activated astrocytes responsible for non-cell autonomous neurotoxicity to motor neurons.

2. Materials and methods

2.1. Experimental animals

Transgenic (Tg) female rats overexpressing an ALS-linked mutant human Cu/Zn superoxide dismutase gene (*SOD1*) were used (Nagai et al., 2001). The Tg rats ubiquitously express His46Arg mutant human *SOD1* in the heterozygous state. The ALS Tg rats were divided into three groups: presymptomatic (approximately 20–21 weeks of age, no symptoms, $n = 16$), early symptomatic (approximately 24–25 weeks of age, mild unilateral hindlimb paresis, $n = 16$), and late symptomatic stage (approximately 28–29 weeks of age, complete paraplegia, $n = 16$). Age-matched non-transgenic wild-type littermates (non-Tg, $n = 16$ per group) served as controls. All experimental procedures were approved by the Animal Committee of the Tohoku University Graduate School of Medicine (No. 2014MdA-202) and were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978).

2.2. Cerebrospinal fluid (CSF) sample collection

2.2.1. Human CSF collection

Six sporadic ALS (sALS) patients and six disease controls (patients hospitalized at Tohoku University Hospital from 2012 to 2013) were included. Informed written consent was obtained from all human participants for experimental use of the CSF. All procedures in this study were also approved by the Ethics Committee of Tohoku University Graduate School of Medicine (No. 2017–1-652). The patients with ALS were diagnosed according to the revised El-Escorial criteria (Brooks et al., 2000). Participants' ages, sex, disease durations, CSF cell numbers and CSF protein levels are summarized in Supplementary Table S1. CSF was collected by lumbar puncture, and the specimens were immediately centrifuged at 1000 rpm for 15 min at 4 °C. The supernatant was then aliquoted in sterile 2-ml polypropylene tubes and stored at −80 °C.

2.2.2. Rat CSF collection

The ALS Tg and non-Tg rats ($n = 4$ per group, 6 groups) were anesthetized with isoflurane and 1% halothane in a mixture of 30% oxygen and 70% nitrous oxide. After creating a midline incision of the skin over the third to fifth lumbar spinal processes, the fifth lumbar vertebra was laminectomized, and the dura mater was exposed. A pinhole was placed on the dura mater with a 24-gauge needle, and a polyethylene tube (PE10, Becton Dickinson, New Jersey, USA) was inserted into the subarachnoid space. Approximately 100 μl of the rat CSF was collected to the tube and was processed and stored in sterile 0.5-ml polypropylene tubes at −80 °C, along with the aforementioned human CSF.

2.3. Enzyme-linked immunosorbent assay (ELISA)

To examine BMP4 and noggin in CSF, we used ELISA kits (Human BMP4 ELISA Kit [LS-F25876, lot 6133] and Mouse/Human/Rat NOG/Noggin ELISA Kit [LS-F5920, lot 5844] for human CSF, Rat BMP4 ELISA Kit [LS-F26512, lot 6015] and LS-F5920 for rat CSF, Lifespan Biosciences, Washington, USA), according to the manufacturer's instructions. Human CSF samples were not diluted, and rat CSF samples were diluted at 1:10 with dilution buffer. Each sample was run in duplicate, together with freshly prepared standards. The chemiluminescent quantifications for the BMP4 ELISA kits and the absorbance measurements at 450 nm for the noggin ELISA kits were performed using a microplate reader (Varioscan Flash, Thermo Scientific, Massachusetts, USA).

2.4. Vivo-Morpholino® antisense oligonucleotide (ASO)

The antisense sequences for *Rattus norvegicus Bmp4* mRNA and negative control were designed as shown in Supplementary Table S2 (GeneTools, Oregon, USA). All morpholinos were obtained as prequantitated, sterile, salt-free, lyophilized solids in glass vials (GeneTools). Each ASO was reconstituted to a 0.5 mM solution with sterile water and stocked at room temperature (RT) before administration.

2.5. Continuous intrathecal administration

We carefully observed the body weights (BW) and motor functions of the ALS Tg rats every other day, as reported previously (Shijo et al., 2018). We defined the onset of the motor neuron disease as the day we first observed unilateral hind limb paresis. The day after disease onset, recombinant human noggin protein (6057-NG, lot TNT181510A, TNT201601A, TNT1815091, and TNT2317013, R&D Systems, Minnesota, USA) or ASO was administered intrathecally using osmotic pumps (Durect Corporation, California, USA). The preparation of osmotic pumps and their installation via transplant surgery were described previously (Ishigaki et al., 2007). In brief, osmotic pumps (Model number 2002 for noggin and 1003D for ASO, Durect Corporation, California, USA) were filled to capacity with each solution (17.8 μg/kg/day over 14 days for noggin and 0.40 μg/μl, 1.39 μl/h for 3 days for ASO) or vehicle (phosphate buffered saline [PBS] for noggin and physiological saline for ASO) using a filling needle. The quantity of noggin was determined with reference to a previous report (Matsuura et al., 2008). The quantity and rate of ASO administrations were determined