Neuroscience Letters 687 (2018) 216–222

Contents lists available at ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

Research article

Activation of the unfolded protein response in canine degenerative myelopathy

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ARTICLE INFO

Keywords:
Amyotrophic lateral sclerosis
Degenerative myelopathy
Endoplasmic reticulum stress
Superoxide dismutase 1
Unfolded protein response

ABSTRACT

Canine degenerative myelopathy (DM) is an adult-onset progressive and fatal neurodegenerative disorder. Superoxide dismutase 1 (SOD1) mutations have been reported in affected dogs and immunohistochemical analyses revealed the accumulation of mutant SOD1 (E40K) in spinal neurons and astrocytes. Therefore, this disease is regarded as a unique spontaneous large-animal model of SOD1-mediated amyotrophic lateral sclerosis (ALS) in humans. Recent studies reported that endoplasmic reticulum (ER) stress is a key pathomechanism underlying motor neuron death in ALS. The present study demonstrated the up-regulated expression of the ER stress marker GRP78/BiP (BiP) in the spinal cords of DM-affected dogs. Immunohistochemistry of serial spinal cord sections revealed strong BiP expression in microglia and astrocytes in DM compared to normal control dogs, whereas such difference was not observed in spinal neurons. The results of transcriptional analyses of DM spinal tissues showed increased expression levels of apoptosis signal-regulating kinase 1 (ASK1) and spliced X-box binding protein (XBP1s). E40K-transfected Neuro2A cells expressed higher levels of BiP than wild-type SOD1-transfected cells. These results suggest that the activation of the unfolded protein response (UPR) in microglia and astrocytes plays crucial roles in UPR-mediated inflammation in the spinal cords of DM-affected dogs.

1. Introduction

Canine degenerative myelopathy (DM) is an adult-onset progressive neurodegenerative disorder that affects the spinal cord. Most DM-affected dogs are homozygous for the A allele of a superoxide dismutase 1 (SOD1) missense mutation, SOD1:c.118 G > A, which predicts an E40 K amino acid substitution in the SOD1 protein [2]. Although rare, heterozygotes for the SOD1:c.118 G > A mutation also develop DM [25]. A homozygous c.52 A > T missense mutation was more recently identified in the SOD1 gene in a Bernese Mountain Dog, which predicts a T18S amino acid substitution [24]. In humans, familial amyotrophic lateral sclerosis (fALS) is caused by SOD1 gene mutations; therefore, DM was recently regarded as a spontaneous model of fALS [17]. The histopathological features of DM are axonal loss and demyelination of the spinal cord, which occur most profoundly in the caudal thoracic spinal cord. These changes have been detected in all funiculi and involve the somatic sensory, general proprioception sensory, and motor tracts with the dorsolateral part of the lateral funiculus being the most severely affected [13]. Immunohistochemistry with an anti-SOD1 antibody revealed the accumulation of mutant SOD1 proteins in the spinal neurons and astrocytes [2,19]. Another study showed an increased number of activated macrophages in the dorsal portion of the lateral funiculi [13].

Although many previous studies have attempted to elucidate the pathophysiological mechanisms underlying DM, its precise mechanisms remain largely unknown. Endoplasmic reticulum (ER) stress has recently been implicated in various human neurodegenerative diseases including ALS [1]. ER stress is triggered by the accumulation of unfolded proteins within the ER lumen. Cellular responses to ER stress in an attempt to maintain homeostasis are called the unfolded protein response (UPR). In fALS, an interaction between mutant SOD1 and Derlin-1 induces UPR and activates apoptosis signal-regulating kinase 1 (ASK1)-dependent motor neuron death [18]. Additionally, the up-regulated expression of other ER stress markers in the spinal cords of

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https://doi.org/10.1016/j.neulet.2018.09.040
Received 8 February 2018; Received in revised form 19 September 2018; Accepted 21 September 2018
Available online 29 September 2018
0304-3940/

ALS patients and murine models has been reported [1,9]. The cerebrospinal fluid of DM-affected dogs was found to contain high levels of clusterin [20], which exhibits a number of functional activities including a protective role during ER stress-mediated apoptosis [23]. However, the pathological importance of ER stress in DM remains unclear.

The aim of this study was to investigate whether UPR is part of the underlying pathogenesis of DM, similar to ALS. We hypothesized ER stress is strongly induced in the spinal cord of DM affected dogs. We investigated the expression of GRP78/BiP (BiP), a major ER stress marker, as well as various ER stress marker transcriptomes in the spinal cords of DM affected dogs. We also investigated whether E40K-SOD1 proteins induce ER stress and activate UPR in cultured neuronal cells.

2. Materials and methods

2.1. Animals

We reviewed the records of necropsied dogs stored in the laboratory of Veterinary clinical radiology, Gifu University from January 2013 to December 2016. All DM dogs were submitted for necropsy by their owners or by veterinary clinics with the owner's permission. We enrolled dogs necropsied within 24 h of death and histopathologically diagnosed with DM. All of these dogs were confirmed to carry the homozygous c.118 G > A missense mutation (A/A). The dogs in which their signalment was not recorded or died from causes unrelated to DM were excluded. Wild-type SOD1 homozygotes (G/G) that died of causes other than neurological diseases were included as controls. The cervical spinal cords of these dogs were used in the present study. We previously revealed that the degeneration of the thoracic spinal cord is more severe than that of the cervical spinal cord [12]. We considered that the degeneration of the thoracic spinal cords is in terminal phase and not suitable for observation of spinal cells. On the other hand, we considered that the cervical spinal cord reflects the active phase of degeneration. The genotypes of all dogs were evaluated by real-time PCR, as described in a previous study [3]. The use of the animals was approved by the institutional committee (approval number 15001). All procedures were performed in accordance with the guidelines regulating animal use and ethics at Gifu University.

2.2. Immunohistochemistry of spinal cords

In order to investigate whether ER stress is induced in the spinal cords of DM-affected dogs, we performed immunohistochemistry. Formalin-fixed paraffin-embedded sections of cervical spinal cords were prepared from each dog. Sections were incubated with an anti-GRP78/BiP (BiP) antibody (1:200) (Cell Signaling Technology, Charlottesville, U.S.A), anti-Iba1 antibody (1:800) (Wako, Osaka, Japan), or anti-GFAP antibody (1:15) (Dako, Glostrup, Denmark) at 4 °C overnight. Sections were incubated with HRP-conjugated goat anti-rabbit immunoglobulin secondary antibodies at room temperature for 30 min. DAB was used as the chromogen. Tissue sections were counterstained with hematoxylin.

2.3. Measurement of BiP-immunopositive areas in spinal cords

Sections were captured using a microscope equipped with a digital camera (Leica Application Suite 3.4, Leica, Germany) and ×20 magnification images were analyzed using imaging software (Image J ver. 1.50i, National Institutes of Health, U.S.A.). The anti-BiP antibody-positive area was measured. Three randomly selected squares were used for BiP-immunopositive area measurement, in the ventral horn of the gray matter or the lateral funiculus of the white matter.

2.4. Plasmid construction

A mammalian expression plasmid for GFP-FLAG-tagged canine SOD1 at the C terminus (GFP-cWT-SOD1- FLAG or GFP-E40K-SOD1-FLAG) was generated as described previously [16]. A pAcGFP-C1 vector was used as a mock control.

2.5. In vitro UPR assay

The mouse neuroblastoma cell line Neuro2A was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and kept at 37 °C in humidified 5% CO_2/95% air. Vector DNA was transfected 24 h after plating cells. The ER stress inducer tunicamycin (Cayman Chemical, Ann Arbor, MI, U.S.A.) at a final concentration of 2.0 μg/ml or an equal volume of DMSO was added to the cultures.

2.6. Western blot analysis

We investigated *in vitro* whether E40K-SOD1 induces ER stress *per se*. Twenty-four hours after ER stress induction, cells were collected and incubated in TNG-T (50 mM Tris−HCL pH7.4, 150 mM NaCl, 10% glycerol, and 1% Triton X-100) with protease inhibitor cocktail (complete mini, Roche, Basel, Switzerland). Cell lysates were separated on 10% SDS-PAGE gels and transferred to a polyvinylidene difluoride membrane (Immobilon-P membrane, Merck Millipore, Bedford, MA, U.S.A.). The membrane was incubated with an anti-BiP antibody (1:1000) or anti-GFP antibody (Medical & Biological Laboratories, Nagoya, Japan) as primary antibody and HRP-conjugated anti-rabbit IgG (1:5000) (Jackson ImmunoResearch, Pennsylvania, U.S.A.) or HRP-conjugated anti-mouse IgG (1:2500) (Jackson ImmunoResearch, Pennsylvania, U.S.A.) as secondary antibodies. Immunoreactive proteins on the membrane were visualized with the ECL HRP substrate (Chemi-Lumi one super, Nacalai Tesque, Kyoto, Japan). The band intensities of each blot were quantified using image analysis software (ImageQuant TL ver.8.1, GE Healthcare Japan, Tokyo, Japan).

2.7. Quantitative real-time RT-PCR

We performed quantitative real-time RT-PCR on ER stress-associated genes in order to assess the signaling pathways of UPR in the spinal cords of DM dogs. Total RNA was isolated from cervical cords. Two-step real-time RT-PCR was performed in order to quantify the transcription of 9 canine genes associated with ER stress: BiP, ASK1, CHOP, caspase12, EDEM1, ATF4, XBP1 total (XBP1t), XBP1 unspliced form (XBP1u), and XBP1 spliced form (XBP1s). Primer sequences were previously reported [14]. Three reference genes (SDHA, TBP, and RPL32) were selected as housekeeping genes among 8 candidate genes (GAPDH, HPRT1, EF-1α, SDHA, TBP, HMBS, RPL13 A, and RPL32). All samples were examined in duplicate, and the mean value of ΔCt was calculated. The transcription levels (relative quantity) of each mRNA were calculated by 2-ΔCt, resulting in an n-fold difference relative to those of the mean value of three reference genes. The sequences of the primers used in the present study are listed in Table 1.

2.8. Statistical analyses

Regarding immunohistochemistry and quantitative real-time RT-PCR, all data were analyzed using the Mann-Whitney U test. The confidence interval was 95% and significance was set at $p < 0.05$. Statistical analyses were performed using commercial software (JMP 10.0 program, SAS Institute, Cary, NC, U.S.A).

Detailed methods are described in Supplemental methods.