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Edaravone is a candidate agent for spinal muscular atrophy: *In vitro* analysis using a human induced pluripotent stem cells-derived disease model

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ABSTRACT

Spinal muscular atrophy (SMA) is an intractable disease characterized by a progressive loss of spinal motor neurons, which leads to skeletal muscle weakness and atrophy. Currently, there are no curative agents for SMA, although it is understood to be caused by reduced levels of survival motor neuron (SMN) protein. Additionally, why reduced SMN protein level results in selective apoptosis in spinal motor neurons is still not understood. Our purpose in this study was to evaluate the therapeutic potential of edaravone, a free radical scavenger, by using induced pluripotent stem cells from an SMA patient (SMA-iPSCs) and to address oxidative stress-induced apoptosis in spinal motor neurons. We first found that edaravone could improve impaired neural development of SMA-iPSCs-derived spinal motor neurons with limited effect on nuclear SMN protein expression. Furthermore, edaravone inhibited the generation of reactive oxygen species and mitochondrial reactive oxygen species up-regulated in SMA-iPSCs-derived spinal motor neurons, and reversed oxidative-stress induced apoptosis. In this study, we suggest that oxidative stress might be partly the reason for selective apoptosis in spinal motor neurons in SMA pathology, and that oxidative stress-induced apoptosis might be the therapeutic target of SMA.

1. Introduction

Spinal muscular atrophy (SMA) is an inherited autosomal recessive disease characterized by progressive skeletal muscle atrophy and weakness with spinal motor neuron degeneration (Crawford and Pardo, 1996). SMA is mainly caused by lack of functional SMN protein, which is coded by two highly homologous genes: survival motor neuron 1 (*SMN1*) gene and *SMN2* gene on chromosome 5q11.2–13.3 in humans (Coovert et al., 1997; Hahnen et al., 1995; Lefebvre et al., 1995). *SMN1* transcripts almost completely include exon 7 (FL-SMN), but the majority of *SMN2* transcripts lack exon 7 (Δ7-SMN) on alternative splicing (Lorson et al., 1999). Therefore, when the *SMN1* gene is mutated or deleted, much of the SMN protein produced by only *SMN2* transcripts is unstable Δ7-SMN, which leads to SMA pathology (Jodelka et al., 2010).

Many researchers have developed treatment strategies, which can be classified into an “increasing SMN strategy” and a “non-SMN targeting strategy”. However, no critical, reliable, and efficacious strategies are thus far established in the therapy of SMA, and symptomatic treatment such as rehabilitation and respiratory care are provided in clinical practice.

We previously established a human *in vitro* SMA-iPSCs model with cells from a patient with SMA type III to develop a cure for SMA, as some stem cell-based research groups reported (Ebert et al., 2009; Sareen et al., 2012; Wang et al., 2013). Using this *in vitro* disease model, we observed efficacy of the thyrotropin-releasing hormone (TRH) analog, 5-oxo-L-prolyl-L-histidyl-L-prolinamide, for SMA. However, treatment with the TRH analog could not reverse the SMA pathology in increased caspase 3-positive apoptotic cells (Ohuchi et al., 2016).

Oxidative stress-induced apoptosis is related to pathology in some neurodegenerative disorders such as Parkinson's disease, Alzheimer's disease, and amyotrophic lateral sclerosis (ALS) (Blesa et al., 2015; Perry et al., 2002; Tohgi et al., 1999). Moreover, the development of antioxidants for treatment of these diseases has been eagerly anticipated. In fact, edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one), a free radical scavenger, showed efficacy in not only acute brain infarction (Nishi et al., 1989) but also ALS (Ito et al., 2008; Yoshino and Kimura, 2006). On the other hand, there have been few reports focusing on the relationship between SMA and oxidative stress. A previous report stated that SMN knockdown resulted in increased mitochondrial membrane potential and increased free radical production in NSC-34 mouse motor

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neuron-like cells (Acsadi et al., 2009). In addition, 4-hydroxy-2-nonenal-modified protein, a product of membrane lipid oxidation, reportedly increased in the spinal motor neurons in some SMA patients (Hayashi et al., 2002). Therefore, more detailed studies are needed on the potential of antioxidants, in particular edaravone, for treatment of SMA.

Herein, we demonstrate increase of oxidative stress-induced apoptosis in differentiated SMA-iPSCs and the potential efficacy of edaravone for SMA by using a human *in vitro* SMA-iPSCs model. These results suggest that oxidative stress-induced apoptosis is one of the therapeutic targets of SMA, and edaravone may be a potential agent for SMA treatment.

2. Material and methods

2.1. Ethics statement

The establishment and pathological analysis of patient-derived iPSCs in this study were approved by the Ethics Review Committee of the National Hospital Organization, Nagara Medical Center, and informed consents were obtained from the parents of our pediatric patients. During this study, the established human stem cells were handled according to the protocols for clinical research using human stem cells of the National Hospital Organization, Nagara Medical Center.

2.2. iPSCs culture and spinal motor neuron differentiation

The iPSC colonies were maintained using primate ES cell medium (ReproCELL, Kanagawa, Japan) supplemented with 4 ng/ml basic fibroblast growth factor (Wako, Osaka, Japan) and 500 U/ml penicillin/streptomycin (PS, Life Technologies, Carlsbad, CA, USA). The iPSC colonies were cultured in 5% CO_2 at 37 °C and passaged every 7 days.

For spinal motor neuron differentiation from iPSCs, we used a previously reported protocol (Ohuchi et al., 2016). Briefly, we placed 5000 single iPSCs into a primary differentiation medium containing Dulbecco's modified Eagle medium (DMEM)/F12 (Life Technologies), 5% Knockout Serum Replacement (KSR, Life Technologies), and 500 U/ml PS with 2 μM dorsomorphin (Sigma-Aldrich, St. Louis, MO, USA), 10 μM SB431542 (SB, Cayman, San Diego, CA, USA) and 10 μM Rho-associated coiled-coil forming kinase inhibitor, Y-27632 (Wako). The medium was changed to the primary differentiation medium with 2 μM dorsomorphin and 10 μM SB431542 after 3 days. The spheres were then plated onto Matrigel-coated 96-well plates (Becton, Dickinson and Company, NJ, USA) in primary differentiation medium supplemented with 2 μM dorsomorphin and 10 μM SB431542 for another 7 days. The neural precursor cells were then cultured in the second differentiation medium containing DMEM/F12, 1% N2 supplement (Life Technologies), and 500 U/ml PS supplemented with 0.1 μM retinoic acid (RA, Sigma Aldrich). The cells were then cultured in the second differentiation medium with 1 μM purmorphamine (PMN, Miltenyi Biotec, Bergisch, Gladbach, Germany) and 0.1 μM RA for 7 days. Finally, the generated spinal motor neurons were cultured in supplemented second differentiation medium containing 10 ng/ml brain-derived neurotrophic factor (BDNF, R & D Systems Inc., Minneapolis, MN, USA), 10 ng/ml glial cell line-derived neurotrophic factor (GDNF, R & D), 1 μM cyclic adenosine monophosphate (cAMP, Wako) and 200 ng/ml ascorbic acid (AA, Sigma-Aldrich). In all differentiation stages, the medium was changed every 2 or 3 days.

2.3. Drug assay

Fibroblast cells were exposed to 3-methyl-1-phenyl-2-pyrazolin-5-one (edaravone, Sigma-Aldrich) for 24 h. The differentiated spinal motor neurons were exposed to the edaravone for 42–56 days of our induction protocol by changing the medium every 3 days. Then, these cultured cells were washed with phosphate buffered saline (PBS, Life Technologies) three times and collected for each assay.

2.4. Immunocytochemistry

After the plated cells were washed with PBS three times, they were fixed in 4% paraformaldehyde (Nacalai Tesque, Kyoto, Japan) for 20 min at 4 °C and then washed again with PBS three times. The cells were blocked with 5% donkey serum and 0.1% Triton X-100 (Nacalai Tesque) in PBS for 30 min at 4 °C. The cells were washed with PBS three times, and incubated with primary antibodies overnight at 4 °C. After washing two times, the cells were labeled with the appropriate secondary antibody-tagged fluorescent dye. Nuclear staining was performed using Hoechst 33342 (Life Technologies). The primary antibodies are listed in Supplemental Table 1 and the secondary Alexa Flour-labeled antibodies used include 594 donkey anti-mouse IgG, 488 donkey anti-mouse IgG, and 488 donkey anti-rabbit IgG (Life Technologies, dilution for all second antibody is 1:1000). Images were taken by BIOREVO BZ-9000 (Keyence, Osaka, Japan) and the images of the fibroblast taken from an SMA patient at high magnification were taken by a confocal microscope (FLUOVIEW FV10i; Olympus, Tokyo, Japan).

To analyse nuclear SMN expression, we measured the SMN protein positive area per Hoechst positive area using BIOREVO BZ-9000.

2.5. Western blot analysis

Briefly, cells lysed using radioimmunoprecipitation assay buffer were centrifuged at 12,000*g* for 10 min, and the supernatants were analysed. Protein concentrations were determined using a bicinchoninic acid assay protein assay kit (Pierce Biotechnology, Rockford, IL, USA) with bovine serum albumin as a standard. Equal volumes of the lysate and sample buffer containing 20% 2-mercaptoethanol (Wako) were mixed and the samples were boiled for 5 min at 100 °C. Then the proteins were separated using 5–20% SDS-polyacrylamide gel electrophoresis, and were transferred to an Immun-Blot polyvinylidene fluoride membrane (Bio-Rad Laboratories, Hercules, CA, USA). The primary antibodies used for immunoblotting in this study are listed in Supplemental Table 1 and the secondary antibodies were goat anti-mouse and goat anti-rabbit IgGs conjugated to horseradish peroxidase (Thermo Fisher Scientific, Waltham, MA, USA, dilution for all second antibody is 1:2000). The immunoreactive bands were visualized using ECLTM prime western blotting detection reagent (GE Healthcare, Buckinghamshire, UK). Band intensities were measured using the ImageQuant LAS-4000 mini-image analyser (GE Healthcare) and detected band analysis software (Multi Gauge; Fujifilm, Tokyo, Japan).

2.6. Cellular reactive oxygen species detection assay

The cellular reactive oxygen species was detected with DCFDA cellular reactive oxygen species detection assay kit (Abcam, Cambridge, MA, USA). After washing with PBS, cell clumps were harvested using trypsin and incubated for 30 min with 20 μM DCFDA at 37 °C. The cells were analysed using a BD FACS Canto II (Becton, Dickinson and Company). DCF was excited by the 488 nm laser and detected at 535 nm, and the ratio of reactive oxygen species production cells was determined.

2.7. MitoSOX

We used MitoSOX™ Red mitochondrial superoxide indicator for live-cell imaging (Life Technologies) to detect mitochondrial superoxide. After washing the plated cells with PBS, cells were incubated with 5 μM MitoSOX for 10 min at 37 °C in the dark. Then the plated cells were washed with PBS three times again and we measured the MitoSOX positive area by BIOREVO BZ-9000.