Stem Cell Research 15 (2015) 459–468

Contents lists available at ScienceDirect

Stem Cell Research

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

Amyotrophic lateral sclerosis models derived from human embryonic stem cells with different superoxide dismutase 1 mutations exhibit differential drug responses

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ARTICLE INFO

Article history:
Received 15 September 2015
Accepted 15 September 2015
Available online 24 September 2015

Keywords:
Amyotrophic lateral sclerosis
SOD1
Human embryonic stem cell
Motor neuron
Drug response

ABSTRACT

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative motor neuron (MN) disease. The gene encoding superoxide dismutase 1 (SOD1) is a causative element of familial ALS. Animal ALS models involving SOD1 gene mutations are widely used to study the underlying mechanisms of disease and facilitate drug discovery. Unfortunately, most drug candidates have failed in clinical trials, potentially due to species differences among rodents and humans. It is unclear, however, whether there are different responses to drugs among the causative genes of ALS or their associated mutations. In this study, to evaluate different SOD1 mutations, we generated SOD1-ALS models derived from human embryonic stem cells with identical genetic backgrounds, except for the overexpression of mutant variants of SOD1. The overexpression of mutant SOD1 did not affect pluripotency or MN differentiation. However, mutation-dependent reductions in neurite length were observed in MNs. Moreover, experiments investigating the effects of specific compounds revealed that each ALS model displayed different responses with respect to MN neurite length. These results suggest that SOD1 mutations could be classified based the response of MNs to drug treatment. This classification could be useful for the development of mutant-specific strategies for drug discovery and clinical trials.

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease characterized by death of both upper and lower motor neurons (MNs) (Beckman et al., 2001). About 90% of ALS cases are sporadic. The remaining 10% of cases are classified as familial ALS (FALS), which is characterized by mutations in genes including superoxide dismutase 1 (SOD1), transactive response DNA-binding protein 43 kDa (TARDBP, TDP-43), fused in sarcoma (FUS) and chromosome 9 open reading frame 72 (C9ORF72) (Rosen et al., 1993) (Neumann et al., 2006) (Vance et al., 2009) (Renton et al., 2011). Approximately 20% of FALS cases are caused by mutations in the SOD1 gene, and over a hundred SOD1 mutations have been reported to date (Renton et al., 2014). The average age at onset of SOD1-related ALS (SOD1-ALS) is 55 years; the primary hallmark of both SOD1-ALS and other forms of the disease is death of MNs leading to muscle weakness (Lyall et al., 2001) (Millecamps et al., 2010) (Bruijn et al., 2004). Riluzole is the drug approved for the treatment of ALS, and is effective in the extension of the patient's lifetime for a relatively limited duration. Hence, there is a great demand for more efficacious drugs. Although many candidate drugs for ALS have been discovered using animal models, almost all of these drugs have failed clinical trials, potentially because of species differences in drug responses among rodents and human beings (Glicksman, 2011) (Limpert et al., 2013).

Human pluripotent stem cell (hPSCs)-based disease models are promising tools to overcome the problems associated with species differences. Recently, studies reported cellular ALS models that used hPSCs including human embryonic stem cells (hESCs) and induced pluripotent stem cell (iPSCs). Using these models, MN death was observed, indicating an ALS phenotype (Wada et al., 2012) (Bilican et al., 2012). Additionally, new drug candidates, kenpaullone and anacardic acid were discovered (Egawa et al., 2012; Yang et al., 2013). However, because these models do not generally have an identical genetic

Abbreviations: ALS, amyotrophic lateral sclerosis; cAMP, cyclic adenosine monophosphate; dbcAMP, dibutyryl-cAMP; DMSO, dimethyl sulfoxide; hESCs, human embryonic stem cells; hPSCs, human pluripotent stem cell; HPRT1, hypoxanthine phosphoribosyltransferase 1; iPSCs, induced pluripotent stem cell; MN, motor neuron; MRB, mutants and metal-binding region; ROCK, Rho-associated protein kinase; RT-PCR, reverse transcription polymerase chain reaction; SOD1, superoxide dismutase 1; SOD1-hESCs, SOD1-overexpressing hESCs; WT, wild-type.

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http://dx.doi.org/10.1016/j.scr.2015.09.006

background, it is difficult to accurately compare results among the various mutations in ALS-related genes.

Here, to investigate the specific differences among SOD1 mutations, we generated hESC-derived models of SOD1-ALS with identical genetic backgrounds, except for those resulting in the overexpression of SOD1 variants. We found no differences in pluripotency or the ability to differentiate into MNs among any of the SOD1-overexpressing hESCs (SOD1-hESCs). Our cell model also demonstrated mutant-specific phenotypes including mutant-dependent reduction in MN neurite length. Additionally, using several compounds, we found that each ALS model showed different responses to these compounds.

2. Materials and methods

2.1. Establishment of SOD1-overexpressing hESC lines

HESCs were cultured on mitomycin-C-treated mouse embryonic fibroblasts in primate ES medium (ReproCELL, Japan), supplemented with 5 ng/mL fibroblast growth factor 2 (Wako Chemicals, Japan), as previously described (Suemori et al., 2006). To generate WT or mutant SOD1 overexpressing hESCs, the site-specific gene integration method, which we developed previously (Sakurai et al., 2010), was applied. Both the SOD1 and Cre recombinase expression vectors were introduced into the parental hESCs derived from the hESC line KhES-1 (Suemori et al., 2006). SOD1 cDNAs in the expression vector were integrated into the HPRT1 locus on the genome of the parental hESCs. Hygromycin-resistant clones, which showed normal cell growth, were isolated and used as SOD1-overexpressing hESCs (SOD1-hESCs). Karyotype analysis was carried out by Nihon Gene Research Laboratories, Inc. The hESC lines were used in accordance with the Guidelines for Derivation and Utilization of Human Embryonic Stem Cells proposed by the Ministry of Education, Culture, Sports, Science, and Technology (MEXT) of Japan.

2.2. Immunocytochemistry

Cells were fixed in both 4% paraformaldehyde (Nacalai Tesque, Japan) and 4% sucrose (Wako), and permeabilized using 0.2% Triton X-100 (Nacalai Tesque, Japan). After blocking with 1.5% bovine serum albumin at room temperature, cells were incubated with a primary antibody overnight, followed by a secondary antibody for 1 h at 4 °C. The primary antibodies used in this study were mouse anti-OCT3/4 monoclonal antibody (mAb) (Santa Cruz Biotechnology), rabbit anti-NANOG polyclonal antibody (pAb; Cell Signaling Technology), mouse anti-SSEA4 mAb (Millipore), mouse anti-TRA-1–60 mAb (Millipore), rabbit anti-nestin pAb (Millipore), mouse anti-OLIG2 mAb (Millipore), mouse anti-ISL-1/2 mAb (Developmental Studies Hybridoma Bank), mouse anti-MNR2 mAb (HB9, Developmental Studies Hybridoma Bank), mouse anti-TUJ1 mAb (Sigma-Aldrich), and rabbit anti-MAP2 pAb (Millipore). Secondary antibodies used in this study were goat anti-mouse immunoglobulin-G pAb conjugated with Alexa Fluor 488 or 546 (Molecular Probes), and goat anti-rabbit immunoglobulin-G pAb conjugated with Alexa Fluor 488 or 546 (Molecular Probes). Cells were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) for visualization of all nuclei after the treatment of secondary antibody. Image acquisition was performed using an inverted Olympus IX71 epi-fluorescence microscope with an Olympus DP73 CCD camera (Olympus). Images were randomly captured from more than six or ten fields per single experiment to calculate MN differentiation rates or for measuring MN morphologies, respectively. The neurite length and soma sizes of neurons were measured by CellSens Dimension software (Olympus).

2.3. Western blot analysis

Confluent hESCs were dissolved with cold RIPA buffer (Sigma) supplemented with halt protease and phosphatase inhibitor cocktail (Thermo Scientific) and mixed using a vortex mixer. Lysates were centrifuged at 12,000 rpm for 20 min at 4 °C and the supernatants were used as protein samples. Protein concentrations were measured using the Pierce BCA Protein Assay Kit (Thermo Scientific). Protein samples were diluted with 4× Laemmli Sample Buffer (Bio Rad) and 2-mercaptoethanol (Wako) and then boiled at 95 °C for 5 min. Samples were electrophoresed through 4–20% precast gel (Cosmo Bio Co., Japan) with Tris/Glycine/SDS Buffer (Bio Rad), and then transferred to polyvinylidene difluoride membranes (Bio Rad) with Tris/Glycine Buffer (Bio Rad). After treatment with Blocking One solution (Nacalai Tesque), membranes were incubated with a primary antibody overnight at 4 °C and then incubated with a secondary antibody for 1 h at room temperature. To visualize proteins bonding with antibodies, the Novex ECL HRP Chemiluminescent Substrate Reagent Kit (Invitrogen) and the luminescent image analyzer LAS3000 (Fujifilm) were used. Quantification of the band intensity was performed by Multi Gauge software (Fujifilm). The primary antibodies used in this study were rabbit anti-superoxide dismutase1 pAb (Abcam), rabbit anti-superoxide dismutase pAb (Stressgen), and mouse anti-β-actin mAb (Abcam). The secondary antibodies used were anti-mouse IgG HRP-linked antibody (Cell Signaling), goat anti-rabbit IgG HRP-linked antibody (Santa Cruz).

2.4. Motor neuron differentiation

Two MN differentiation methods were carried out in this study (Supplementary Fig. S2). The first was our previous method with a slight modification (Wada et al., 2012; Wada et al., 2009) (Supplementary Fig. S2A). Briefly, a bone morphogenetic protein inhibitor, LDN193189 (200 nM; Cellagen Technology), was used as a neural inducer (stage P1, shown in Supplementary Fig. S2A). In stage P2, cells were treated with 200 nM LDN193189 and 100 nM all-trans retinoic acid (ATRA; Sigma-Aldrich) in N2B27 neural differentiation medium (NDM). Neural rosettes in the P2 cells were re-plated on poly-L-lysine/laminin/fibronectin (PLL/LM/FN)-coated culture dishes and cultured in NDM supplemented with 2.5 μM ATRA, 1 μM purmorphamine (Cayman Chemical), and 2.5 μM CHIR99021 (R&D). After 2 days, the medium was changed to NDM supplemented with 1 μM ATRA, 1 μM purmorphamine (Tocris Bioscience), and 1 μM CHIR99021, and cells were cultured for 5 days. Then, the cells were cultured in NDM containing 10 ng/mL brain-derived neurotrophic factor (BDNF; PeproTech), 10 ng/mL glial cell-derived neurotrophic factor (GDNF; PeproTech), and 10 ng/mL neurotrophin-3 (NT-3; PeproTech) for another 2 weeks.

The second differentiation method was established in this study (Supplementary Fig. S2B). For neural induction, dissociated ES colonies were grown on PLL/LM-coated culture dishes in NDM supplemented with 200 nM LDN193189 and 1 μM SB431542 for 8 days (stage P1, shown in Supplementary Fig. S2B). The medium was changed every other day. Primary colonies were split into small groups of cells using 200 U/mL collagenase with 1 mM $CaCl_2$, plated on new PLL/LM-coated culture dishes, and cultured in NDM supplemented with 200 nM LDN193189 (stage P2, shown in Supplementary Fig. S2B) for 8 days. Then, cells were re-plated and cultured in NDM alone for 8 days. Neural rosettes formed during the P3 stage. Neural rosettes were dissociated using Accutase (Innovative Cell Technologies, Inc) and plated onto gelatin-coated culture dishes for 45–60 min at 37 °C to remove non-neural cells. Unattached cells were then cultured on PLL/LM/FN-coated culture dishes in NDM supplemented with 0.5 μM ATRA, 0.5 μM purmorphamine, 0.5 μM CHIR99021, 10 ng/mL BDNF, 10 ng/mL GDNF, and 10 ng/mL NT-3 for 7 days. For long-term culture in the P4 stage, cells were treated 1 μM Cytarabine (AraC), and the medium was changed every 7 days.

2.5. Chemicals

Kenpaullone and riluzole were purchased from Cayman Chemical. Y27632 and dbcAMP were purchased from Wako Chemicals. TRO19622