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Lysosomal dysfunction and early glial activation are involved in the pathogenesis of spinocerebellar ataxia type 21 caused by mutant transmembrane protein 240

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ABSTRACT

Spinocerebellar ataxia type 21 (SCA21) is caused by missense or nonsense mutations of the transmembrane protein 240 (TMEM240). Molecular mechanisms of SCA21 pathogenesis remain unknown because the functions of TMEM240 have not been elucidated. We aimed to reveal the molecular pathogenesis of SCA21 using cell and mouse models that overexpressed the wild-type and SCA21 mutant TMEM240. In HeLa cells, overexpressed TMEM240 localized around large cytoplasmic vesicles. The SCA21 mutation did not affect this localization. Because these vesicles contained endosomal markers, we evaluated the effect of TMEM240 fused with a FLAG tag (TMEM-FL) on endocytosis and autophagic protein degradation. Wild-type TMEM-FL significantly impaired clathrin-mediated endocytosis, whereas the SCA21 mutants did not. The SCA21 mutant TMEM-FL significantly impaired autophagic lysosomal protein degradation, in contrast to wild-type. Next, we investigated how TMEM240 affects the neural morphology of primary cultured cerebellar Purkinje cells (PCs). The SCA21 mutant TMEM-FL significantly prevented the dendritic development of PCs, in contrast to the wild-type. Finally, we assessed mice that expressed wild-type or SCA21 mutant TMEM-FL in cerebellar neurons using adeno-associated viral vectors. Mice expressing the SCA21 mutant TMEM-FL showed impaired motor coordination. Although the SCA21 mutant TMEM-FL did not trigger neurodegeneration, activation of microglia and astrocytes was induced before motor miscoordination. In addition, immunoblot experiments revealed that autophagic lysosomal protein degradation, especially chaperone-mediated autophagy, was also impaired in the cerebella that expressed the SCA21 mutant TMEM-FL. These dysregulated functions in vitro, and induction of early gliosis and lysosomal impairment in vivo by the SCA21 mutant TMEM240 may contribute to the pathogenesis of SCA21.

1. Introduction

Spinocerebellar ataxia type 21 (SCA21) is an early onset autosomal dominant neurological disease with a slow progression of cerebellar ataxia. SCA21 is characterized by mental retardation and cognitive impairment (Vuillaume et al., 2002; Delplanque et al., 2008). Delplanque et al. recently demonstrated that SCA21 is caused by missense or nonsense mutations of transmembrane protein 240 (TMEM240)

Abbreviations: SCA21, spinocerebellar ataxia type 21; TMEM240, transmembrane protein 240; LAMP2A, lysosome-associated membrane protein 2a; TSG101, tumor susceptibility gene 101; Iba1, ionized calcium-binding adapter molecule 1; GFAP, glial fibrillary acidic protein; LC3, microtubule associated protein 1 light chain 3; MEF2D, myocyte enhancer factor 2D; Hsc70, heat shock cognate protein 70; GAPDH,;DIV, ays in vitro; TMR, tetramethylrhodamine; OG, Oregon green; PFA, paraformaldehyde; HT, HaloTag; FL, FLAG tag; MVBs, multivesicular bodies; LAMP1, lysosomal-associated membrane protein 1; Tf, transferrin; MA, macroautophagy; mA, microautophagy; CMA, chaperone-mediated autophagy; AAV, adeno-associated virus; PC, Purkinje cell; DCN, deep cerebellar nuclei; ML, molecular layer; PCL, Purkinje cell layer; GCL, granule cell layer; PKCγ, protein kinase Cγ; MAP kinase, mitogen-activated protein kinase; ESCRT, endosomal sorting complexes required for transport; BGC, Bergmann glial cell; DAMPs, damage-associated molecular patterns

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(Delplanque et al., 2014). TMEM240 is a protein with two membrane-spanning domains and has been detected in mouse synaptosomal preparations (Trinidad et al., 2012). *TMEM240* mRNA is highly expressed in the human brain compared to other tissues according to several gene expression databases (Human Protein Atlas (http://www.proteinatlas.org/, (Uhlén et al., 2015)), Expression Atlas (https://www.ebi.ac.uk/gxa/home, (Kapushesky et al., 2012)), RefEx (http://refex.dbcls.jp/) and GTEx portal (http://www.gtexportal.org/home/, (Carithers et al., 2015))). These findings suggest that TMEM240 has a role in the regulation of brain functions. However, no report has investigated the molecular properties and cellular functions of TMEM240. In addition, it is difficult to predict the molecular functions of TMEM240 because TMEM240 does not have homology to other proteins. In this study, we attempted to demonstrate the subcellular localization and cellular functions of TMEM240 that was overexpressed in HeLa cells and primary cultured neurons. TMEM240 is involved in the regulation of endocytosis in HeLa cells, and in neurite elongation in primary cortical neurons. SCA21 mutations abolished these functions. Moreover, SCA21 mutations impaired autophagic lysosomal protein degradation in HeLa cells, and the dendritic development of cerebellar Purkinje cells (PCs), whereas wild-type TMEM240 did not affect these phenomena. These dysregulated functions of mutant TMEM240 may contribute to the pathogenesis of SCA21. Furthermore, model mice that expressed the SCA21 mutant TMEM240 in cerebellar neurons showed impaired motor coordination, accompanied by early activation of microglia and astrocytes. In addition, impairment of autophagic lysosomal protein degradation, especially chaperone-mediated autophagy, was also observed in mouse cerebella expressing SCA21 mutant TMEM240. These findings suggested that motor dysfunction caused by cerebellar expression of the mutant TMEM240 is mediated by lysosomal impairment and early glial activation, which could be related to the pathogenesis of SCA21.

2. Materials and methods

2.1. Materials

Human *TMEM240* cDNA was obtained from GE Healthcare (Chicago, IL, USA). Anti-β-actin mouse monoclonal, anti-FLAG mouse monoclonal, anti-CD63 mouse monoclonal, anti-microtubule associated protein 1 light chain 3 (LC3) rabbit polyclonal, anti-lysosome-associated membrane protein 2a (LAMP2A) rabbit polyclonal, anti-beclin 1 rabbit polyclonal, anti-tumor susceptibility gene 101 (TSG101) rabbit polyclonal, anti-β-actin rabbit polyclonal, and anti-Rab18 rabbit polyclonal antibodies were obtained from Sigma-Aldrich (St. Louis, MO, USA). BODIPY 493/503 (difluoro{2-[1-(3,5-dimethyl-2H-pyrrol-2-ylidene-N)ethyl]-3,5-dimethyl -1H-pyrrolato-N}boron was also obtained from Sigma-Aldrich. Neuron culture medium, neuron dissociation solution, bafilomycin A1, and anti-ionized calcium-binding adapter molecule 1 (Iba1) rabbit polyclonal antibody were obtained from Wako Pure Chemicals (Osaka, Japan). Penicillin/streptomycin solution was purchased from Nacalai Tesque (Kyoto, Japan). The HaloTag system (HaloTag vector, HaloTag ligand and anti-HaloTag mouse monoclonal antibody) was obtained from Promega (Madison, WI, USA). Fluorescent dye (Alexa488 or Alexa555)-conjugated goat anti-rabbit or anti-mouse IgG antibodies, neurobasal medium, B-27 serum-free supplement, Lipofectamine 3000, and transferrin-Alexa568 were supplied by Thermo Fisher Scientific (Waltham, MA, USA). Anti-LAMP1 mouse monoclonal (H4A3) antibodies were obtained from the Hybridoma Bank (Iowa City, IA, USA). Anti-calbindin rabbit monoclonal, anti-Bip rabbit monoclonal, anti-Rab7 rabbit monoclonal, anti-glial fibrillary acidic protein (GFAP) rabbit monoclonal, anti-p38 rabbit polyclonal, and anti-phospho-p38 (Thr180/Tyr182) rabbit polyclonal antibodies were purchased from Cell Signaling Technologies (Danvers, MA, USA). Anti-myocyte enhancer factor 2D (MEF2D) mouse monoclonal antibody was obtained from BD Biosciences (San Jose, CA, USA). Anti-p62 rabbit polyclonal and anti-Atg5 rabbit polyclonal antibodies were supplied by Medical & Biological Laboratories (Nagoya, Japan). Anti-C1orf70 (TMEM240) goat polyclonal antibody was obtained from Santa Cruz Biotechnology (Dallas, TX, USA). Anti-cathepsin D goat polyclonal antibody was purchased from R&D systems (Minneapolis, MN, USA). Anti-heat shock cognate protein 70 (Hsc70) mouse IgM antibody was obtained from Novus Biologicals (Littleton CO, USA). Anti-TIP47 rabbit polyclonal antibody was supplied by ProteinTech (Rosemont, IL, USA). Glass-bottomed culture dishes (35-mm diameter) were obtained from MatTek (Ashland, MA, USA). Plasmids to express GFP-Rab5 and GFP-Rab7 were kindly gifted by Prof. Mitsunori Fukuda (Tohoku University, Japan). Plasmids to express LC3 were generously gifted by Prof. Noboru Mizushima (University of Tokyo, Japan).

2.2. Construction of plasmids and adeno-associated viral vectors

Human *TMEM240* cDNA fused with HaloTag or FLAG-tag, or without any tags, was subcloned into pcDNA5/FRT (Thermo Fisher Scientific) for transient expression in HeLa cells. *TMEM240* cDNA fused with a FLAG-tag was subcloned into vectors for the production of adeno-associated virus serotype 9 (AAV9) vectors to be expressed in primary cultured neurons and mouse cerebellar neurons in vivo. Missense or nonsense mutations identified in SCA21 were introduced using a PrimeStar mutagenesis basal kit (Takara Bio, Kusatsu, Japan). AAV9 vectors were constructed as described previously (Konno et al., 2014).

2.3. Cell culture and transfection

HeLa cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 100 units/mL of penicillin, and 100 mg/mL of streptomycin in a humidified atmosphere containing 5% CO2 at 37 °C. HeLa cells were transfected with plasmids to express TMEM240 fused with HaloTag (TMEM-HT), TMEM240 fused with FLAG (TMEM-FL), or untagged TMEM240, using Lipofectamine 3000. Four hours after transfection, cells were detached with a trypsin-EDTA solution and spread onto 12-well plates for immunoblotting and also onto glass-bottom dishes for fluorescent imaging.

Rat cerebellar primary cultures were prepared using the Nerve-Cell Culture System/Dissociation Solution, as previously described (Seki et al., 2009). Briefly, the cerebella of E16 embryos from pregnant Wistar/ST rats were dissociated using the Dissociation Solution. Dissociated cells were cultured in the Nerve-Cell Culture Medium (serum free) supplemented with 100 pM 3,3′,5-triiodo-L-thyronine for 21 days in vitro (DIV). The cells were infected with AAV9 vectors to express GFP and TMEM-FL on DIV1.

2.4. SCA21 model mice

Cerebellar injection of AAV9 vectors was conducted as previously described (Huda et al., 2014). We injected AAV9 vectors (8×10^9 vg) to express GFP, wild-type, or R116C mutant TMEM-FL under a neuron-specific synapsin I promoter into the cerebella of 4-week-old male C57BL/6 J mice. The motor performance of the mice was examined by the beam-walking test (Hijioka et al., 2017) 1, 3, 5, 7, 9, and 11 weeks after viral injection. Mice were placed on a beam (1.1 m length, 1.5 cm width and 50 cm height). Usage of the hind limb during beam walking was evaluated as a fault rate.

Mice were sacrificed 3 and 12 weeks after viral injection for immunostaining and immunoblotting. For immunostaining, mice were fixed by perfusion with 4% paraformaldehyde (PFA). The whole brain was removed from the skull, followed by post-fixation. For immunoblotting, mice cerebella were isolated from the skull after perfusion with PBS and frozen at −80 °C.