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Polyglutamine-expanded ataxin-7 activates mitochondrial apoptotic pathway of cerebellar neurons by upregulating Bax and downregulating Bcl-x_L

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Abstract

Spinocerebellar ataxia type 7 (SCA7) is an autosomal dominant neurodegenerative disorder caused by polyglutamine-expanded ataxin-7. In the present investigation, we expressed disease-causing mutant ataxin-7-Q75 in the primary neuronal culture of cerebellum with the aid of recombinant adenoviruses. Subsequently, this in vitro cellular model of SCA7 was used to study the molecular mechanism by which mutant ataxin-7-Q75 induces neuronal death. TUNEL staining studies indicated that polyglutamine-expanded ataxin-7-Q75 caused apoptotic cell death of cultured cerebella neurons. Mutant ataxin-7-Q75 induced the formation of active caspase-3 and caspase-9 without activating caspase-8. Polyglutamine-expanded ataxin-7-Q75 promoted the release of apoptogenic cytochrome-*c* and Smac from mitochondria, which was preceded by the downregulation of Bcl-x_L protein and upregulation of Bax protein expression in cultured cerebellar neurons. Further real-time TaqMan RT-PCR assays showed that mutant ataxin-7-Q75 upregulated Bax mRNA level and downregulated Bcl-x_L mRNA expression in the primary neuronal culture of cerebellum. The present study provides the evidence that polyglutamine-expanded ataxin-7-Q75 activates mitochondria-mediated apoptotic cascade and induces neuronal death by upregulating Bax expression and downregulating Bcl-x_L expression of cerebellar neurons.
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1. Introduction

Spinocerebellar ataxia type 7 (SCA7) is an autosomal dominant neurological disorder and caused by CAG trinucleotide repeat expansion within the coding region of *SCA7* gene [1–3]. Both mRNA and protein of ataxin-7, *SCA7* gene product, are widely distributed in the brain and peripheral tissues [4,5]. Normally, ataxin-7 contains 4–35 glutamines at the N-terminal domain, and polyglutamine tract of disease-causing ataxin-7 expands to 37–306 glutamines [3,4]. Wild-type ataxin-7 protein is expressed in both cytoplasm and nucleus of human CNS neurons [5]. Despite the wide distribution of ataxin-7 in the brain, SCA7 neurodegeneration is only prominent in the cerebellum, pons, inferior olive, anterior horn of spinal cord and retina [3,6].

SCA7 is one of nine known polyglutamine neurological disorders including Huntington's disease [3,7]. Recent investigations suggested that neuronal death observed in polyglutamine disorders resulted from the activation of apoptotic pathway [8,9]. It has been shown that polyglutamine-containing N-terminal huntingtin or expanded poly-

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glutamine repeats induced apoptotic cell death by activating caspase-3, caspase-8 and caspase-9 [10,11]. Caspase activation and apoptotic neuronal death were found in transgenic mouse model of Huntington's disease [12–14]. An increased level of activated caspases was observed in postmortem brain tissue of patients affected with Huntington's disease [14–16]. The involvement of apoptosis in mutant ataxin-7-induced neurodegeneration was suggested by previous studies showing that number of activated caspase-3 positive neurons was increased in human SCA7 brain and that apoptotic cell death was observed in the retina of SCA7 knockin mice [17,18]. However, the molecular mechanism by which polyglutamine-expanded ataxin-7 activates caspase-3 and apoptotic pathway remains to be answered.

In previous investigations, an in vitro cellular model of SCA7 was prepared by expressing wild-type and polyglutamine-expanded ataxin-7 proteins in various cell lines [17,19]. However, mutant polyglutamine ataxin-7 causes neurotoxic effects in a certain subset of CNS neurons, indicating that cell-specific mechanism is involved in mutant ataxin-7-induced neuronal death in the brain. Thus, CNS neurons, which are vulnerable to polyglutamine-expanded ataxin-7-induced neurotoxicity in vivo, should be used to study the molecular mechanism underlying mutant ataxin-7-induced neuronal death. In the present study, we expressed polyglutamine-expanded ataxin-7-Q75 in the primary neuronal culture of cerebellum, which undergoes neurodegeneration in SCA7 brain, with the aid of recombinant adenoviruses. Subsequently, this in vitro cellular model of SCA7 was used to investigate the molecular mechanisms by which mutant ataxin-7-Q75 induces neuronal death. Our results suggest that polyglutamine-expanded ataxin-7-Q75 causes apoptotic death of cerebellar neurons by upregulating Bax level and down-regulating Bcl-x_L expression.

2. Materials and methods

2.1. Preparation of primary cultured neurons

Primary neuronal culture of cerebellum or neocortex was prepared as described previously [20]. Briefly, cerebellum or neocortex was dissected from rats at the postnatal day 1 or 2 and incubated for 30 min at 37 °C in DMEN/F12 medium containing pronase (0.5 mg/ml, Roche) and DNase I (0.3 mg/ml, Roche). Tissue fragments were subsequently triturated, and dissociated cells were plated onto poly-L-ornithine-coated six-well dishes. CNS neurons were cultured in DMEN/F12 medium supplemented with 3% fetal bovine serum (HyClone), N1 nutrient supplement (Sigma), 50 U/ml penicillin and 50 μg/ml streptomycin. From the second day in culture, proliferation of glial cells was prevented by the addition of uridine and 5′-fluoro-2′-deoxyuridine.

2.2. Preparation of recombinant adenoviruses

Full-length cDNAs encoding human wild-type ataxin-7-Q10 and polyglutamine-expanded ataxin-7-Q75 were kindly provided by Dr. Harry Orr of University of Minnesota [19]. According to our previous study [21], influenza hemagglutinin epitope (HA, YPYDVPDYA) was added to the C-terminus of ataxin-7-Q10 or ataxin-7-Q75 by performing PCR amplification. AdEasy Adenoviral Vector System (Stratagene) was used to prepare recombinant adenoviruses by homologous recombination [22]. Briefly, cDNA of ataxin-7-Q10 or ataxin-7-Q75 was subcloned into pShuttle vector provided in the kit. The resulting plasmid was linearized and cotransformed into E. coli. BJ5183 cells with adenoviral backbone plasmid pAdEasy-1. Recombinant adenoviral plasmid was then transfected into HEK 293 cells, which provide *E1* gene required for the production of infectious viral particles. Following the amplification in HEK 293 cells, viral stocks were purified by CsCl gradient ultracentrifugation [23] and titered using Adeno-X rapid titer kit (Clontech). As a control, we also prepared recombinant adenoviruses containing cDNA of GFP. Twenty-four hours after plating, cultured neurons were infected with recombinant adenovirus at multiplicity of infection (MOI) of 20–30 pfu/cell.

2.3. Immunoblotting analysis of human ataxin-7 expressed in cultured neurons

Following infecting cultured neurons with adenoviruses containing cDNA of ataxin-7-Q10 or ataxin-7-Q75, protein lysate was obtained by homogenizing neurons with SDS sample buffer. Protein samples were fractionated on 8% SDS-polyacrylamide and transferred to polyvinylidene difluoride (PVDF) membrane. Then, PVDF membrane was incubated with diluted polyclonal anti-HA antiserum (Santa Cruz) or monoclonal anti-polyglutamine 1C2 antibody (Chemicon) overnight at 4 °C. After being washed, membrane was incubated with sheep anti-mouse or donkey anti-rabbit horseradish peroxidase-linked secondary antibody (Amersham). Subsequently, immunoreactive proteins were visualized by using enhanced chemiluminescence protocol (ECL Kit, Amersham).

2.4. Analysis of neuronal death

Following the expression of ataxin-7-Q10 or ataxin-7-Q75, cell death was analyzed by performing MTT assay and TUNEL (Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) staining. For MTT assay, cultured cerebellar or neocortical neurons were incubated with 3-(4,5-dimetylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 0.2 mg/ml) for 2 h at 37 °C. Subsequently, neurons were washed, and formazan released was quantified at 560 nm by using an ELISA plate reader.

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