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Overexpression of HGF attenuates the degeneration of Purkinje cells and Bergmann glia in a knockin mouse model of spinocerebellar ataxia type 7

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ABSTRACT

Spinocerebellar ataxia type 7 (SCA7) is an autosomal dominant disorder associated with cerebellar neurodegeneration caused by expansion of a CAG repeat in the ataxin-7 gene. Hepatocyte growth factor (HGF), a pleiotrophic growth factor, displays highly potent neurotrophic activities on cerebellar neurons. A mutant c-met/HGF receptor knockin mouse model has revealed a role for HGF in the postnatal development of the cerebellum. The present study was designed to elucidate the effect of HGF on cerebellar neurodegeneration in a knockin mouse model of SCA7 (SCA7-KI mouse). SCA7-KI mice were crossed with transgenic mice overexpressing HGF (HGF-Tg mice) to produce SCA7-KI/HGF-Tg mice that were used to examine the phenotypic differences following HGF overexpression. The Purkinje cellular degeneration is thought to occur *via* cell-autonomous and non-cell autonomous mechanisms mediated by a reduction of glutamate transporter levels in Bergmann glia. The Purkinje cellular degeneration and reduced expression of glutamate transporters in the cerebellum of SCA7-KI mice were largely attenuated in the SCA7-KI/HGF-Tg mice. Moreover, phenotypic impairments exhibited by SCA7-KI mice during rotarod tests were alleviated in SCA7-KI/HGF-Tg mice. The bifunctional nature of HGF on both Purkinje cells and Bergmann glia highlight the potential therapeutic utility of this molecule for the treatment of SCA7 and related disorders.

1. Introduction

Spinocerebellar ataxia type 7 (SCA7) is a progressive inherited disorder characterized by ataxia and neurodegeneration of the cerebellum and retina (Ieraci et al., 2002). The disease is the result of an abnormal CAG repeat expansion in the ataxin-7 gene. SCA7 patients display ataxia in addition to neurodegeneration and neuronal death of Purkinje cells (Ieraci et al., 2002). SCA7 knockin mice also show a neurodegeneration of Purkinje cells (Yoo et al., 2003). Therefore, it is thought that the protection of Purkinje cells may represent a therapeutic strategy to combat SCA7.

Additionally, following findings suggest that Bergmann glial cells have been considered as another therapeutic target of the disease. Bergmann glia are cerebellum-specific astrocytes that are located around synapses between Purkinje cells and cerebellar granule cells or climbing fibers. The glial cells play a role in removing excess glutamate from synapses *via* two primary glutamate transporters, glutamate/aspartate transporter (GLAST) and glutamate transporter-1 (GLT-1) (Huang and Bordey, 2004). A mouse model that expresses expanded ataxin-7 specifically in Bergmann glia displays a neurodegeneration of Purkinje cells, indicating that a dysfunction of Bergmann glia contributes to the degeneration of Purkinje cells in SCA7 mice and thereby progression of the disease in a non-cell autonomous manner (Custer et al., 2006). Therefore, prevention of cell degeneration and concomitant increase of glutamate transporter function may represent a valid therapeutic strategy for SCA7.

Hepatocyte growth factor (HGF), which was first identified as a potent mitogen for mature hepatocytes (Nakamura et al., 1984, 1989), exhibits neurotrophic activities in a wide variety of neurons in the hippocampus, the cerebral cortex, the cerebellum, the brainstem (midbrain dopaminergic neurons) and the spinal cord (sensory and motor neurons) (Funakoshi and Nakamura, 2011). Recent experiments have indicated that HGF exerts neuroprotective effects on various neurons in animal models of cerebral ischemia, amyotrophic lateral sclerosis (ALS) and spinal cord injury (Funakoshi and Nakamura, 2011; Sun et al., 2002; Ishigaki et al., 2007; Kitamura et al., 2011; Miyazawa et al., 1998). In the cerebellum, HGF is expressed in Purkinje cells and granular cells, and plays a role in the cerebellum during both developmental and adult

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stages (Honda et al., 1995; Ieraci et al., 2002). In a mutant with a partial loss of Met function, the cerebellum was smaller than in controls and showed abnormal foliation (Ieraci et al., 2002). In addition to the cell growth and development, HGF exhibits neuroprotective effects for mature granule cells in primary cerebellar neuron culture (Zhang et al., 2000; Hossain et al., 2002). Moreover, overexpression of HGF not only attenuates the degeneration of motor neurons as a neurotrophic factor but also maintains adequate levels of the astrocytic glutamate transporter GLT-1 in a transgenic mouse model of ALS (Sun et al., 2002). This evidence led us to hypothesize that HGF may have a therapeutic potential on cerebellar neurons and Bergmann glia, cerebellar astrocyte subpopulations, in a valid mouse model of SCA7 in which a targeted 266 CAG repeat segment (a length known to cause infantile disease onset) of ataxin-7 is knocked into the mouse *ATXN7*/*Sca7* locus. These mice show features, which resemble those observed in an infantile SCA7 patient (Yoo et al., 2003).

The purpose of this study was to examine the effect of HGF on the Purkinje cells and Bergman glia of SCA7-KI mice. For this purpose, transgenic mice overexpressing HGF in a neuron-specific manner (HGF-Tg mice; Sun et al., 2002) were crossed with SCA7 knockin mice (SCA7-KI mice; Yoo et al., 2003) and phenotypic comparisons were made in wild-type (WT), HGF-Tg, SCA7-KI, and SCA7-KI/HGF-Tg mice. Overexpression of HGF attenuated the shrinkage of Purkinje cells and prevented reduction of glutamate transporters in Bergmann glia and improved motor performance during the rotarod test in SCA7-KI mice.

2. Materials and methods

2.1. Animals

The SCA7 knockin ($Sca7^{266Q/5Q}$: SCA7-KI) mouse is a knockin mouse, which is a valid model of SCA7 that contains a targeted insertion of 266 CAG repeats (a number that causes infantile-onset disease) into the mouse *Sca7* locus. The mice were generously provided by Dr. Huda Zoghbi from the Baylor College of Medicine, Houston, TX (Yoo et al., 2003). Neuron-specific enolase (NSE)-promoter driven HGF transgenic (HGF-Tg) mice were generated and maintained as previously described (Sun et al., 2002). Heterozygous SCA7-KI male mice were crossed with heterozygous HGF-Tg female mice, which had been backcrossed with C57BL/6J mice for more than seven generations, to generate WT, heterozygous HGF-Tg, heterozygous SCA7-KI, and heterozygous SCA7-KI/HGF-Tg mice. Mouse genotypes were determined by dot blot hybridization or by polymerase chain reaction (PCR) using forward (5′-TTGTAGGAGCGGAAAGAATGTC-3′) and reverse (5′-CCACCCACAGATTCCACGAC-3′) primers for SCA7-KI and with forward (5′-CCAAACATCCGAGTTGGTTACT-3′) and reverse (5′-ATTACAACTTGTATGTCAAAAT-3′) primers for HGF-Tg mice. Experimental protocols were approved by the Animal Experimentation Ethics Committee of Asahikawa Medical University and Osaka University Graduate School of Medicine. All efforts were made to minimize animal discomfort and the number of animals used.

2.2. Cerebellar neuronal culture

Sixteen-day-old mouse embryos (E16) were obtained from timed pregnant C57BL/6J females (Japan SLC, Hamamatsu, Japan) that had been deeply anesthetized with isoflurane and euthanized *via* decapitation. Routinely, two pregnant females were processed in parallel. Immediately after euthanasia, uteri containing the embryos were removed and transferred into a sterile 100 mm tissue culture dish that was kept on ice and filled with ~20 ml ice-cold Leibovitz's L-15 medium. The cerebella were dissected using a stereomicroscope. After removing the meninges, the isolated cerebellar primordia were minced and transferred to a 15-ml Falcon tube containing L-15 medium. The supernatant was replaced with a pre-warmed 0.25% trypsin solution and the cerebella were incubated for 4–5 min at 37 °C with gentle shaking. Incubation was terminated by the addition of fetal bovine serum (JRH Biosciences, Brooklyn, Australia). Following the addition of DNase I and centrifugation, cells were dissociated by repeated pipetting and separated from non-dissociated tissue by sedimentation. The cells were seeded in plates precoated with poly-L-ornithine (500 μg/ml) at 2.5×10^5 cells/cm^2. Cultures were grown in neurobasal medium (Gibco Invitrogen, Grand Island, NY) supplemented with B27 (Gibco Invitrogen), 2 mM GlutaMaxI (Gibco Invitrogen), 1 mM adenine, 3 mM KCl, 1% heat-inactivated horse serum (Gibco Invitrogen), and a mixture of penicillin–streptomycin (100 U/ml and 100 μg/ml; Nacalai Tesque, Kyoto, Japan). From 2 days after seeding, 10 μM triiodothyronine (T3) and 1 μM Ara-C were added in order to mature Purkinje cells and to prevent the proliferation of non-neuronal cells. Half of the medium was replaced with fresh medium every 2 days. The cultures were maintained at 37 °C in a humidified incubator with 5% CO_2 and 95% air. The cells cultured for 14 days were washed with phosphate buffered saline (PBS) and fixed with 10% formalin in PBS.

2.3. Tissue preparation

Animals (WT, HGF-Tg, SCA7-KI, and SCA7-KI/HGF-Tg mice) at 10 weeks of age ($n = 3$ each) were deeply anesthetized with sodium pentobarbital and transcardially perfused with ice-cold PBS followed by ice-cold 4% paraformaldehyde in PBS. The cerebella were excised and immersed in the same fixative for several hours at 4 °C. Fixed tissues were immersed in 10% sucrose in PBS overnight at 4 °C, followed by 20% sucrose in PBS for 6 h at 4 °C, after which they were subsequently frozen in powdered dry ice or CO_2 gas. Frozen tissues were cut into either 16-μm or 40-μm thick sagittal sections using a Leica CM3050 S or CM1900 cryostat (Leica Microsystems GmbH, Wetziar, Germany).

2.4. Immunocytochemistry and immunohistochemistry

Formalin-fixed cerebellar neurons or cryosections were incubated in blocking buffer consisting of 10% normal goat serum (S26-100 mL, CHEMICON, Temecula, CA) and 0.3% Triton X-100 in PBS for an hour at room temperature followed by one or two of the following primary antibodies for 20 h at 4 °C: (1) mouse monoclonal anti-calbindin D28K antibody (1:250; 300, Swant, Marly, Switzerland); (2) mouse monoclonal anti-GFAP (glial fibrillary acidic protein) antibody (1:250; MAB3402, CHEMICON); (3) rabbit polyclonal anti-GFAP antibody (1:10; N150687, DAKO, Glostrup. Denmark); (4) rabbit polyclonal anti-c-Met antibody (1:50; SP260, Santa Cruz Biotechnology, Santa Cruz, CA); (5) rabbit polyclonal anti-rat HGF antibody (Ohya et al., 2007; Yamada et al., 1995) (6) guinea-pig polyclonal anti-GLAST antibody (1:500; AB1782, CHEMICON); (7) guinea-pig polyclonal anti-GLT-1 antibody (1:600; AB1783, CHEMICON). For immunostaining of phospho-c-Met, sections were incubated with Blocking One Histo (Nacalai Tesque) for an hour at room temperature, and then immunoreacted with rabbit polyclonal anti-phosphorylated c-Met antibody (1:200; C7240, Sigma, St. Louis, MO) in Signal Enhancer HIKARI B Solution (Nakalai Tesque) for 20 h at 4 °C. After washing the sections with PBS, immunoreactivity was visualized by incubating them further for 20 min at room temperature with secondary antibodies conjugated with Alexa Fluor 488, Alexa Fluor 546 or Alexa Fluor 647 diluted 1:600 (Invitrogen, Carlsbad, CA). Fluorescence-immunostained sections were observed under an Olympus FV1000