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Gene therapy for a mouse model of glucose transporter-1 deficiency syndrome

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ABSTRACT

Objective: We generated an adeno-associated virus (AAV) vector in which the human *SLC2A1* gene was expressed under the synapsin I promoter (AAV-h*SLC2A1*) and examined if AAV-h*SLC2A1* administration can lead to functional improvement in *GLUT1*-deficient mice.
Methods: AAV-h*SLC2A1* was injected into heterozygous knock-out murine *Glut1* (GLUT1$^{+/-}$) mice intraperitoneally (systemic; 1.85×10^{11} vg/mouse) or intra-cerebroventricularly (local; 1.85×10^{10} vg/mouse). We analyzed GLUT1 mRNA and protein expression, motor function using rota-rod and footprint tests, and blood and cerebrospinal fluid (CSF) glucose levels.
Results: Vector-derived RNA was detected in the cerebrum for both injection routes. In the intracerebroventricular injection group, exogenous GLUT1 protein was strongly expressed in the cerebral cortex and hippocampus near the injection site. In the intraperitoneal injection group, exogenous GLUT1 protein was mildly expressed in neural cells throughout the entire central nervous system. The motor function test and CSF/blood glucose ratio were significantly improved following intra-cerebroventricular injection.
Conclusions: AAV-h*SLC2A1* administration produced exogenous GLUT1 in neural cells and improved CSF glucose levels and motor function of heterozygous knock-out murine Glut1 mice.

1. Introduction

Glucose transporter 1 deficiency syndrome (GLUT1DS; OMIM #606777) is an autosomal dominant disorder caused by haploinsufficiency of *SLC2A1*, the gene encoding GLUT1. GLUT1 is expressed in the central nervous system (CNS), mainly in the endothelial cells of the blood-brain barrier, but also in other types of cells including neurons [1–4]. GLUT1 interacts with other glucose transporters and mediates glucose transport into neurons [2,3,5]. Heterozygous mutation of *SLC2A1* leads to impaired hexose transport into the brain, resulting in irreversible neurologic dysfunction [6–8]. The biochemical hallmark for GLUT1DS is hypoglycorrhachia: cerebrospinal fluid (CSF) glucose level < 40 mg/dL and CSF/blood glucose ratio < 0.45 [8,9].

The classical symptoms of GLUT1DS are intractable seizures, intellectual disability, ataxia, and dystonia starting at infancy. In adulthood, some GLUT1DS patients develop paroxysmal exercise-induced dyskinesia [10]. The current and major therapy for GLUT1DS is a ketogenic diet, which is a high-fat, carbohydrate-restricted diet [11,12]. A ketogenic diet is effective for seizures, transient aggravation after fasting, and ataxia [12]. In addition, a modified Atkins diet may be effective for seizures and partially effective for cognitive function [13]. However, a ketogenic diet is not effective for the intellectual disability and movement disorder observed in adult patients with GLUT1DS [7,10,12] and can lead to hyperlipidemia and is considered a primary risk factor for the development of atherosclerosis [14]. The other treatment for GLUT1DS, triheptanoin, which is a medium chain triglyceride with odd chain fatty acids, has the potential to improve the paroxysmal motor disorder [15], but its long-term efficacy is unknown.

Recently, gene therapy has given remarkable results in various clinical trials and mouse models [16,17]. With its low immunogenicity and long-term expression in non-dividing post-mitotic neuronal cells, the

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adeno-associated viral vector (AAV) appears to be an optimal vehicle for the treatment of congenital CNS disorders [17]. Therefore, we aimed to develop gene therapy for GLUT1DS. To this end, we investigated if *SLC2A1* gene delivery using an AAV vector can lead to functional improvement in *GLUT1* - heterozygous knock-out murine mice.

2. Materials and methods

2.1. Glut1-deficient mice

Mice with heterozygous knock-out of the murine *Glut1* gene (GLUT1$^{+/-}$ mice) were generated by the Department of Molecular Biopharmacy and Genetics, Graduate School of Pharmaceutical Sciences, Tohoku University, Sendai, Japan [18]. The gene mutation of GLUT1$^{+/-}$ mice was created by the insertion of a gene-trapping vector that contains a splice acceptor site followed by a neomycin resistance (neo) gene with a polyadenylation signal in intron 1 of the *Glut1* gene. GLUT1$^{+/-}$ mice have microcephaly, impaired motor activity, epileptic discharges on electroencephalography, hypoglycorrhachia, and decreased brain glucose uptake by positron emission tomography scanning [18,19]. GLUT1$^{+/-}$ mice mimic the classical phenotype of human patients with GLUT1DS, and GLUT1$^{-/-}$ mice were embryonic lethal. All animal studies were approved by the Animal Care Committee, Jichi Medical University (approval number, 16-192).

2.2. Generation of AAV vectors

The AAV vector plasmids contained an expression cassette consisting of the neuron-specific synapsin I promoter, followed by human *SLC2A1*, sharing high homology with mouse GLUT1 (96% identical at the amino acid level) with or without myc-DDK (FLAG®), and a simian virus 40 polyadenylation signal sequence between the inverted terminal repeats of the AAV3 genome (Fig. 1). We synthesized the AAV9 *vp* cDNA as described previously [20] with the substitution of thymidine for adenine 1337, which introduced an amino acid change from tyrosine to phenylalanine at position 446. Recombinant AAV vectors were produced by transient transfection into HEK293 cells using the vector plasmid, an AAV3 *rep* and AAV9 *vp* expression plasmid, and the adenoviral helper plasmid pHelper (Agilent Technologies, Santa Clara, CA). We purified the recombinant viruses by isolation from two sequential continuous CsCl gradients, and viral titers were determined by quantitative PCR.

2.3. Infection of neuronal SH-SY5Y cells with AAV-hSLC2A1

The human neuroblastoma SH-SY5Y cell line was seeded in an 8-well chamber slide (0.8 cm^2/well at 1.0×10^5 cells/well). After an overnight incubation at 37 °C, the cells were infected with AAV-h*SLC2A1* at 3.7×10^9 vg/well. At 40 h after infection with AAV-h*SLC2A1*, the SH-SY5Y cells were fixed with 4% paraformaldehyde for 15 min. After 3 washes with phosphate-buffered saline (PBS) for 5 min, the fixed cells were incubated with blocking buffer (PBS containing 4% goat serum, 0.1% Triton X-100) for 1 h at room temperature (RT) and incubated with primary antibodies (containing blocking buffer) overnight at 4 °C. The following primary antibodies were used: mouse monoclonal anti-c-myc (9E10) (sc-40; Santa Cruz Biotechnology, Santa Cruz, CA) at 1:150 and rabbit polyclonal anti-GLUT1 (N-terminus) (TA301678; OriGene Technologies, Rockville, MD) at 1:100. After 3 washes with 0.1% Triton X-100, immune-complexes were detected using the following secondary antibodies (for 2 h at RT): goat anti-mouse IgG Alexa Fluor 594 (Invitrogen, Carlsbad, CA) at 1:250 and goat anti-rabbit IgG Alexa Fluor 488 (Invitrogen, Carlsbad, CA) at 1:250. Hoechst 33342 was used to visualize nuclei. Confocal images were acquired with a FluoView™ FV1000 confocal microscope (Olympus, Tokyo, Japan).

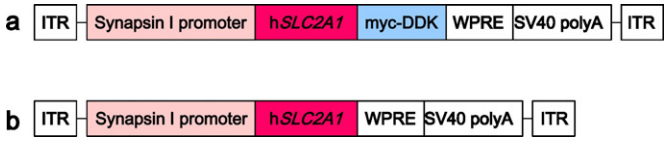

Fig. 1. Structure of AAV-h*SLC2A1*. We selected the neuron-specific synapsin I promoter for the expression of *SLC2A1* in neural cells with a reduced off-target effect [20,24,25]. The human *SLC2A1* sequence was fused with a myc-DDK (FLAG®) tag at its C-terminus (a). We also created a tag sequence-removed AAV-h*SLC2A1* to assess its clinical application (b). ITR: inverted terminal repeat; WPRE: woodchuck hepatitis virus posttranscriptional regulatory element; SV40 polyA: simian virus 40 poly A.

2.4. Systemic or intra-cerebroventricular injection of GLUT1$^{+/-}$ mice with AAV-hSLC2A1

For systemic administration, AAV-h*SLC2A1* (1.85×10^{11} vg; total 50 μL/mouse) was injected into the peritoneum at 7 days after birth, because brain tissue was less damaged at the early neonatal period. For CNS administration, AAV-h*SLC2A1* (1.85×10^{10} vg; total 5 μL/mouse) was injected directly into the bilateral lateral ventricles of the brain at 6 weeks after birth, because patients with GLUT1DS are diagnosed from the infant to childhood period [6–8]. We could not observe any symptoms for GLUT1$^{+/-}$ mice at 6 weeks. Intra-cerebroventricular injections of GLUT1$^{+/-}$ mice were performed using a sharp glass electrode under intraperitoneal 2% chloral hydrate anesthesia. The injection site was measured relative to the bregma: 0.5 mm posterior to the bregma, 1.0 mm laterally from the sagittal suture, and 1.75 mm depth. For control GLUT1$^{+/-}$ mice, saline (total 5 μL) was injected into the bilateral lateral ventricles of the brain at 6 weeks after birth.

2.5. Analysis of exogenous and endogenous GLUT1 mRNA expression by reverse transcriptase-polymerase chain reaction (RT-PCR)

2 months after AAV-h*SLC2A1* injection, mice were sacrificed under CO_2 anesthesia. Total RNA was extracted from the whole cerebrum of mice using the TRIzol® reagent (Invitrogen, Carlsbad, CA). We reverse transcribed 2.5 μg total RNA to cDNA using a SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen, Carlsbad, CA), followed by PCR amplification using TaKaRa Taq™ DNA Polymerase (TAKARA BIO, INC., Otsu, Japan). To confirm the expression of exogenous *GLUT1* mRNA, a reverse primer for vector specific RT-PCR was designed according to the myc-tag sequence: forward primer 5′-ACTGTCGTGTCGCTGTTTG-3′, reverse primer 5′-TGCTGCCAGATCCTCTTCTG-3′. PCR was conducted with the following cycling conditions: 1 cycle at 94 °C for 3 min, 27 cycles at 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s, and a final elongation step at 72 °C for 10 min. Additionally, we confirmed the total amount of *GLUT1* mRNA expression using primers corresponding to both the human *SLC2A1* and murine *Slc2a1* sequences: forward primer 5′-AACTGGGCAAGTCCTTTG-3′, reverse primer 5′-TTCTTCTCCCGCATCATCTG-3′. PCR was conducted with the following cycling conditions: 1 cycle at 94 °C for 3 min, 25 cycles at 94 °C for 30 s, 54 °C for 30 s, and 72 °C for 30 s, and a final elongation step at 72 °C for 10 min. Primers were designed for glyceraldehyde 3-phosphate dehydrogenase (*G3PDH*) mRNA as a quantitative control: forward primer 5′-ACCACAGTCCATGCCATCAC-3′, reverse primer 5′-TCCACCACCCTGTTGCTGTA-3′ (TOYOBO, Osaka, Japan). PCR was conducted with the following cycling conditions: 1 cycle at 94 °C for 3 min, 25 cycles at 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s, and a final elongation step at 72 °C for 10 min.

2.6. Quantitative real-time PCR analysis

Single-strand cDNA was synthesized from 2.5 μg total RNA by reverse transcription. According to the manufacturer's protocol, quantitative real-time PCR was performed using an Applied Biosystems® 7500 Fast Real-Time PCR System (Applied Biosystems, Life Technologies, Carlsbad, CA) with 2× TaqMan® Fast Universal PCR Master Mix, reverse transcribed cDNA, primers, and fluorescently-labeled probes. To confirm the