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Development of a model system for neuronal dysfunction in Fabry disease

Christine R. Kaneski [a,*], Roscoe O. Brady [a], John A. Hanover [b], Ulrike H. Schueler [b]

[a] National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, MD 20892, USA
[b] National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892, USA

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ABSTRACT

Fabry disease is a glycosphingolipid storage disorder that is caused by a genetic deficiency of the enzyme alpha-galactosidase A (AGA, EC 3.2.1.22). It is a multisystem disease that affects the vascular, cardiac, renal, and nervous systems. One of the hallmarks of this disorder is neuropathic pain and sympathetic and parasympathetic nervous dysfunction. The exact mechanism by which changes in AGA activity result in change in neuronal function is not clear, partly due to of a lack of relevant model systems. In this study, we report the development of an *in vitro* model system to study neuronal dysfunction in Fabry disease by using short-hairpin RNA to create a stable knock-down of AGA in the human cholinergic neuronal cell line, LA-N-2. We show that gene-silenced cells show specifically reduced AGA activity and store globotriaosylceramide. In gene-silenced cells, release of the neurotransmitter acetylcholine is significantly reduced, demonstrating that this model may be used to study specific neuronal functions such as neurotransmitter release in Fabry disease.

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1. Introduction

Fabry disease is an X-linked glycosphingolipid-storage disorder caused by a deficiency of the lysosomal enzyme α-galactosidase A (AGA, EC 3.2.1.22). As a consequence of reduced activity of this enzyme, globotriaosylceramide (Gb3), and to a lesser extent, galabiosylceramide, accumulate in the cells of most tissues and organs resulting in a multisystem pathology which frequently includes neurological symptoms [1]. To date, over 600 mutations in the *GLA* gene for AGA expression have been described which result in variable expression of the symptoms of Fabry disease depending on the amount of residual activity in the mutant AGA protein [2].

In its classic form, Fabry disease includes signs and symptoms such as angiokeratoma, corneal clouding, reduced sweating, hearing loss, abdominal pain, diarrhea, neuropathic pain, cardiac hypertrophy, progressive kidney failure, and stroke, and causes significant morbidity even in childhood.

The neurological manifestations of Fabry disease include both peripheral nervous system and CNS involvement [3]. One of the most debilitating symptoms of Fabry disease is severe neuropathic pain that is poorly controlled by currently available pain medications. In addition, the patients experience symptoms of autonomic dysfunction such as reduced sweating, gastrointestinal pain, and changes in gastrointestinal motility and cardiac rhythm [4]. In autopsy findings, autonomic centers throughout the nervous system show increased Gb3 storage by immunohistochemical staining [5]. In skin biopsy studies, Fabry patients show severe loss of intra-epidermal innervation associated with a small-fiber sensory neuropathy [6]. Although enzyme replacement therapy (ERT) with AGA is currently available to treat Fabry disease, even long-term treatment does not completely reverse the neurologic dysfunction found in these patients [7].

The mechanism by which deficiency in AGA activity results in changes in neuronal function is not clear. Neuronal storage of Gb3 is found in dorsal root ganglia neurons in Fabry disease, and it is suggested that this contributes to the peripheral neuropathy [8]. A Gb3 metabolite, globotriaosylsphingosine (lyso-Gb3), which is the deacylated form of Gb3, is dramatically increased in plasma of classically affected male Fabry patients and in plasma and tissues of Fabry mice [9]. These high levels of lyso-Gb3 may also contribute to the pathology of Fabry disease and may be a reason for the painful damage to dorsal root ganglia neurons. Recently, Choi et al. demonstrated that direct application of lyso-Gb3 sensitized nociceptive neurons in the foot pads of normal mice and that lyso-Gb3 increased Ca^{2+} influx in normal dorsal root ganglion cells cultured from adult mice [10]. In a related lysosomal storage disorder, Gaucher disease, we have shown that glucosylsphingosine

Abbreviations: 4-MU, 4-methylumbelliferone; AGA, alpha-galactosidase A; ACh, acetylcholine; B2M, beta-2-microglobulin; CTNF, ciliary neurotrophic factor; FACS, fluorescence activated cells sorting; HEX, hexosaminidase; HRP, horseradish peroxidase; Gb3, globotriaosylceramide; GCS, GemCell™ Calf Serum; lyso-Gb3, globotriaosylsphingosine; pChAT, peripheral acetylcholine transferase; qRT-PCR, quantitative real-time reverse-transcription polymerase chain reaction; RFU, relative fluorescence units; shRNA, short-hairpin RNA; VAChT, vesicular acetylcholine transporter.

* Corresponding author at: National Institutes of Health, Bldg. 8, Room 422, Bethesda, MD 20892, USA.
E-mail address: kaneskic@ninds.nih.gov (C.R. Kaneski).

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(glucopsychosine), an analog of lyso-Gb3, is toxic to cultured neuronal cells [11].

While previous work has documented the accumulation of Gb3 and associated lipids in nervous tissue in autopsy specimens of Fabry patients [5,8] and numerous studies have characterized the small fiber neuropathy and nervous system dysfunctions found in Fabry patients (see [12] for a review), attempts to study these changes on a molecular level have been lacking, in part because a suitable model system is unavailable. Lakoma et al. recently published a study with the Fabry mouse model showing that GLA knock-out mice have many features of peripheral neuropathy found in Fabry patients [13], but there have been no studies with human neuronal systems. With the development of gene silencing techniques, it is now possible to create genetically-engineered cell lines to study the mechanisms of neuronal dysfunction in Fabry disease. To create such an *in vitro* model system, we used gene silencing with short-hairpin RNA to generate a stable knock-down of AGA in LA-N-2, a human neuroblastoma that can be differentiated to neuronal-like cells with characteristics of cholinergic neurons [14].

2. Materials and methods

2.1. Cell culture

LA-N-2 cells were a gift from Dr. J.K. Blusztajn, Boston University, Boston, MA. They were maintained on DMEM/F12 medium supplemented with 15% GemCell Super Calf Serum (GCS, Gemini Bioproducts, Sacramento, CA), 1% non-essential amino acids and 1% penicillin/streptomycin. Growth medium was replaced every 2–3 days, and the cells were subcultured when approximately 80% confluent.

2.2. Gene silencing

Mission™ plasmid shRNA TRCN0000303790 [15] containing a short-hairpin RNA sequence targeting alpha-galactosidase (NM_000169.2) at nucleotide 458, was obtained as a glycerol stock from Sigma-Aldrich Chemical Company, St. Louis, MO. The plasmid contains a sequence-verified shRNA in the TRC2-pLKO-puro vector.

LA-N-2 cells in log-phase growth were seeded in 6-well plates in growth medium. After overnight incubation, cultures were transfected with 2.5 μg purified plasmid complexed with 1.5 μl Omni Ultra-Transfect™ Transfection Reagent (DBio, LLC, Baltimore, MD) according to the manufacturer's directions. After 48 h, the cells were trypsinized and reseeded in a 100 mm dish in growth medium without antibiotics supplemented with 0.6 μg/ml puromycin (selection medium) for 6 days, followed by selection in 1.0 μg/ml puromycin for an additional 7 days. The cells were refed with selection medium every other day. The surviving colonies were expanded in growth medium without puromycin and tested for AGA activity. The colony with the lowest activity (Clone 7) was subcloned by serial dilution and colonies were expanded in selection medium with 3.0 μg/ml puromycin for 13 days.

To control for non-specific effects, LA-N-2 cells were also transfected with a control shRNA plasmid that encodes a scrambled shRNA sequence that will not lead to the specific degradation of any known cellular mRNA. (Control shRNA Plasmid-A, Santa Cruz Biotechnology, Dallas, TX) using the same procedure.

2.3. Neuronal differentiation

For some experiments, LA-N-2 cells were differentiated using a modification of the method of Zineman et al. [16]. Cells were plated in either 2-well glass slides for immunostaining or plastic tissue culture plates at a density of 32,000 cells/cm^2 in growth medium. After overnight incubation to allow the cells to adhere, the cultures were gently washed twice with PBS and refed with Neurobasal™ medium (Gibco) supplemented with glucose (2 mg/ml), GlutaMAX™ (0.5 mM, Gibco), GCS (1%), B27 supplement (2%,Gibco), retinoic acid (3 μg/ml), forskolin (25 μM), recombinant human nerve growth factor (10 ng/ml), CNTF (10 ng/ml), and choline (100 μM). Cultures were incubated for a minimum of 4 days or until the cells stopped proliferating and formed elongated processes. Medium was changed every 2–3 days as necessary.

2.4. Measurement of AGA activity in cell extracts

A standard fluorometric assay for AGA in cell extracts was performed as previously described [17] with modifications. Briefly, cell pellets were resuspended in citrate-phosphate buffer (pH 4.6) containing sodium taurocholate (5 mg/ml) and Triton-X 100 (0.1%). The suspensions were frozen (−20 °C) and thawed once, then centrifuged at 5000×*g* for 5 min. Aliquots of the supernatant were incubated for one hour with 4-methylumbelliferyl-alpha-D-galactopyranoside (5 mM, Research Products International, Mount Prospect, IL) in citrate-phosphate buffer (pH 4.6) in the presence of *N*-acetyl-galactosamine (0.1 M, Research Products International), a specific inhibitor of alpha-galactosidase B [18]. At the end of the incubation period, enzyme activity was stopped with glycine buffer (0.1 N, pH 10.6). Fluorescence in samples was determined using a CytoFluor 4000 plate reader (Applied Biosystems, Foster City, CA) with an excitation filter of 360 nm and emission filter of 490 nm. Amount of product formed was determined using 4-MU standards diluted in 0.1 N glycine stop buffer. Protein levels in extracts were determined using the BCA protein assay kit (Pierce, Rockford, IL) according to the manufacturer's instructions. AGA activity was calculated as nmoles 4-MU formed/hour/mg protein and results were compared to untransfected controls included in the same assay.

2.5. Immunostaining and flow cytometry

For immunostaining, LA-N-2 cells were seeded on 2-well glass slides and differentiated. At the end of the incubation they were fixed with 3% paraformaldehyde and stained by indirect immunofluorescence as previously described [19] using saponin as a permeabilizing agent. For Gb3, cells were stained with a mouse monoclonal antibody to Gb3 (BGR 23, Seikagaku Corp, Falmouth, MA) at a dilution of 1:500 and was detected with Alexa Fluor 488 goat anti-mouse IgG (Molecular Probes). For the pan-neuronal marker, PGP 9.5, cells were stained with guinea pig polyclonal anti-PGP 9.5 antibody (GeneTex, Inc., Irvine, CA) at a dilution of 1:500 which was detected with Alexa Fluor 594 goat anti-guinea pig IgG (Thermo Scientific, Rockford, IL). Slides were imaged with a Keyence BZ-9000 fluorescence microscope with a 20× objective. To control for non-specific staining, all immunostaining experiments included a negative control culture that was stained in parallel with only the appropriate secondary antibody (with Alexa Fluor 488 goat anti-mouse IgG or Alexa Fluor 594 goat anti-guinea pig IgG), but no primary antibody.

For flow cytometry for Gb3 staining, undifferentiated cultures were trypsinized, and cell suspensions were fixed with 2% paraformaldehyde and stained as described for slides except the Gb3 antibody was detected with PE-labeled goat F(ab′)2 anti-Mouse IgG (EMD Millipore, Bellerica, MA). As a control for non-specific staining, an aliquot of each cell line was stained in parallel with only PE-labeled goat F(ab′)2 anti-Mouse IgG but no primary antibody. Stained cells were analyzed using a Guava PCA flow cytometer (EMD Millipore). Results were extracted into FACS 2.0 files and graphed using GraphPad Prism 4.0 for Windows (GraphPad Software, La Jolla CA).

2.6. Quantitative RT-PCR

Isolation of total RNA from cultured cells was performed using a PureLink® RNA extraction kit (Ambion). DNA contamination was removed by treating an aliquot of the extracted RNA with RTS DNase kit (MoBio, Carlsbad, CA) according to the manufacturer's instructions. Amount of RNA was quantified using a Nanodrop 1000