Original Article

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Development of cell models for high-throughput screening system of Charcot-Marie-Tooth disease type 1

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Purpose: Charcot-Marie-Tooth disease (CMT) is a peripheral neuropathy mainly divided into CMT type 1 (CMT1) and CMT2 according to the phenotype and genotype. Although molecular pathologies for each genetic causative have not been revealed in CMT2, the correlation between cell death and accumulation of misfolded proteins in the endoplasmic reticulum (ER) of Schwann cells is well documented in CMT1. Establishment of *in vitro* models of ER stress-mediated Schwann cell death might be useful in developing drug-screening systems for the treatment of CMT1.

Materials and Methods: To develop high-throughput screening (HTS) systems for CMT1, we generated cell models using transient expression of mutant proteins and chemical induction.

Results: Overexpression of wild type and mutant peripheral myelin protein 22 (PMP22) induced ER stress. Similar results were obtained from mutant myelin protein zero (MPZ) proteins. Protein localization revealed that expressed mutant PMP22 and MPZ proteins accumulated in the ER of Schwann cells. Overexpression of wild type and L16P mutant PMP22 also reduced cell viability, implying protein accumulation-mediated ER stress causes cell death. To develop more stable screening systems, we mimicked the ER stress-mediated cell death in Schwann cells using ER stress inducing chemicals. Thapsigargin treatment caused cell death via ER stress in a dose dependent manner, which was measured by expression of ER stress markers.

Conclusion: We have developed genetically and chemically induced ER stress models using Schwann cells. Application of these models to HTS systems might facilitate the elucidation of molecular pathology and development of therapeutic options for CMT1.

Key words: Charcot-Marie-Tooth disease, Endoplasmic reticulum stress, Human PMP22 protein, Myelin P0 protein, Thapsigargin.

Introduction

Charcot-Marie-Tooth disease (CMT) is a genetically and clinically heterogeneous disorder of the peripheral nervous system characterized by progressive extremity muscle degeneration and loss of sensory function [1-4]. CMT is divided into many types according to clinical phenotypes, genetic causes, and mode of inheritance. However, it is mainly divided into CMT type 1 (CMT1) and CMT type 2. The former is caused by genetic defects in Schwann cells and the latter is attributed to an anomaly in peripheral neurons [5].

More than 70 genes have been identified as a cause of CMT. Among them are 14 mutations known to cause a malfunction of Schwann cells [http://www.molgen.ua.ac.be/cmtmutations/]. The most prevalent CMT1 mutations occur in peripheral myelin protein 22 (PMP22) and myelin protein zero (MPZ). In addition, duplication or triplication of PMP22 by genetic recombination also causes CMT1 [6,7].

PMP22 and MPZ are myelin components of Schwann cells and their expression is restricted to Schwann cells. The *PMP22* gene encodes a 22-kD protein comprising about 5% of myelin in the peripheral nervous system. It is expressed in the compact portion of essentially all myelinated fibers [8,9]. MPZ is the major structural protein of peripheral myelin and comprises 50% of the protein in the sheath of peripheral nerves. Its function is to stabilize the myelin assembly by linking adjacent lamellae [10,11].

Although the pathological mechanisms of CMT are not well defined, and might be heterogeneous based on the involved genes, the majority of CMT1 caused by PMP22 and MPZ mutations is related to mutant protein accumulation in the endoplasmic reticulum (ER) due to improper folding [12-14]. In fact, 80% of wild type PMP22 protein is retained in the ER due to its structural instability [15]. Thus mutation of both PMP22 and MPZ, and overproduction of PMP22, causes ER stress-mediated cell death of Schwann cells [16,17].

In this context, understanding ER stress caused by PMP22 and MPZ proteins, and targeting the bypass of ER stress-mediated cell death, might be prerequisite for the treatment of CMT1. Here, we generated genetically and chemically induced ER stress models using Schwann cells as a basis for the development of therapeutic options for CMT1.

Materials and Methods

1. Cell culture

Primary Schwann cells were isolated from Sprague Dawley rats at postnatal day 4 according to Brockes et al. [18] with some modification. Isolated Schwann cells were cultured with 1% fetal bovine serum (FBS), 1% penicillin-streptomycin (PS), low glucose Dulbecco's modified eagle medium (DMEM; Biowest, Nuaille, France), N2 supplement (Life Technologies, Rockville, MD, USA), neuregulin, and forskolin (Sigma, St. Louis, MO, USA). The rat Schwann cell line S16 (ATCC, CRL-2941; Manassas, VA, USA) and HEK293 cells were cultured with 10% FBS, 1% PS, and high glucose DMEM. All experiments were conducted according to protocols approved by the Institutional Animal Care and Use Committees of Samsung Medical Center (2014-112-0006).

Table 1. List of primers for generation of wild type and mutant PMP22 and MPZ clones

Experiment	Primer name	Sequence
cDNA cloning	PMP22-F	5'-CCGCCAGAATGCTCCTCCTGTTGCT-3'
	PMP22-R	5'-TTTTCCCTTCCTCCCTTCCC-3'
	MPZ-F	5'-CTATGGCTCCTGGGGCTCCCTC-3'
	MPZ-R	5'-CTATTTCTTATCCTTGCGAGACT-3'
Mutagenesis	PMP22-L16P-F	5'-TCGTCCTCCACGTCGCGGTGCCGGTGCTGCTGTTC-3'
	PMP22-L16P-R	5'-GTGGAGACGAACAGCAGCACCGGCACCGCGACGTGG-3'
	MPZ-V169fs-F	5'-CGGGGGTGTCCTCGGGGTGGGCTGTTGCTGCTGC-3'
	MPZ-V169fs-R	5'-AAAAGCAGCAGCAGCAACAGCCCACCCCGAGGAC-3'
	MPZ-L184fs-F	5'-CGTGGTTCGGTACTGCTGGGCGCAGGCAGGCGGC-3'
	MPZ-L184fs-R	5'-CTGCAGGGCCGCCTGCCTGCGCCCAGCAGTACCG-3'
	MPZ-R185fs-F	5'-GGTTCGGTACTGCTGGCTACCAGGCAGGCGGCCC-3'
	MPZ-R185fs-R	5'-CTCTGCAGGGCCGCCTGCCTGGTAGCCAGCAGTA-3'
	MPZ-S226fs-F	5'-GTATGCAATGCTGGACCACCAAGCAGAAGCACCA-3'
	MPZ-S226fs-R	5'-ACAGCTTTGGTGCTTCTGCTTGGTGGTCCAGCAT-3'

PMP22, peripheral myelin protein 22; MPZ, myelin protein zero.

2. Construction of *PMP22* and *MPZ* genes

To obtain the *PMP22* gene, cDNA was synthesized using Superscript reverse transcriptase (Invitrogen, Carlsbad, CA, USA) from total mRNA from HEK293 cells. Polymerase chain reaction (PCR) was then performed using the cDNA as a template. The *MPZ* gene was amplified from the pCMV6-entry-MPZ vector (Origene, Rockville, MD, USA). The amplified PCR product was cloned into an expression vector, pCMV-myc (Clontech, Mountain View, CA, USA). Mutant genes were generated using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). All primer sequences are listed in Table 1.

3. Transfection of cloned genes

To express wild type and mutant *PMP22* and *MPZ* genes, S16 and HEK293 cells were transfected with *PMP22* and *MPZ* containing vectors using Lipofectamine 3000 reagent (Invitrogen) according to the manufacturer's protocol. After overexpression of the genes for 48 hours, cells were harvested for further investigation.

4. Measurement of cell viability

HEK293 cells (4×10^4) seeded on 24 well plates were transfected with the indicated amounts of wild type and L16P mutant PMP22 DNA for 72 hours. Rat primary Schwann or S16 cells (3×10^3) seeded on 96 well plates were treated with ER stress inducers such as calcium ionophore A23187 (CI), brefeldin A (BFA), thapsigargin (TG), or tunicamycin (TM; Sigma) for 24 hours. Then cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, cells treated with ER stress inducers were incubated with 10 mM MTT solution for 2 hours then lysed with dimethyl sulfoxide. Relative numbers of viable cells were determined using absorbance at 560 nm.

5. Western blotting

Expression of *PMP22* and *MPZ* proteins were confirmed using standard Western blotting. HEK293 or S16 cells (3×10^5) were transfected with expression vectors then total cell lysate was harvested with RIPA lysis buffer (Thermo Scientific, Rockford, IL, USA). After quantification, proteins were separated and transferred onto polyvinylidene fluoride membranes. Anti-myc (Abcam, Cambridge, UK), anti-actin, anti-mouse secondary, and anti-rabbit secondary antibodies (Sigma), as well as ECL plus Western blotting substrate (Thermo Scientific), were used for detection of proteins. To determine ER stress induced by either overexpression of the proteins or by ER stress inducing chemicals, anti-heat shock 70 kDa protein 5 (BiP), anti-DNA damage inducible transcript 3 (CHOP), and anti-eukaryotic translation initiation factor 2-alpha kinase 3 (PERK) antibodies (Cell Signaling Technology, Danvers, MA, USA) were used. Anti-poly (ADP-ribose) polymerase 1 (PARP1) antibody (Cell Signaling Technology) was also used to determine apoptotic cell death.

6. Immunocytochemistry

Mutant protein cellular localization was determined by immunocytochemistry using anti-myc antibody (Abcam) after transfection of PMP22-L16P and MPZ- R185fs vectors into S16 cells. 4',6-diamidino-2-phenylindole (DAPI) and anti-protein disulfide isomerase (PDI) antibody (Cell Signaling Technology) were used for nuclear and ER detection, respectively. Then myc-tagged proteins were visualized by fluorescent secondary antibodies (Life Technologies).

Results

1. Generation of genetically induced ER stress models

To generate an ER stress model in Schwann cells, we cloned the most prevalent PMP22 and MPZ mutations. We selected the L16P mutation in *PMP22*, and V169fs, L184fs, R185fs, and S226fs mutations in *MPZ*. To effectively detect expression of the proteins, a myc-tag was fused to the N-terminus of each protein. Expression of the mutant proteins, as well as wild type proteins, was determined using standard Western blotting after transfection of the vectors into HEK293 cells (Fig. 1). To determine whether expression of PMP22 mutant proteins induced ER stress, we compared PERK and BiP expression levels. Overexpression of both wild type and L16P mutant PMP22 elevated ER stress markers in a dose dependent manner (Fig. 1A). On the other hand, expression of wild type MPZ did not change the level of ER stress markers (Fig. 1B). Responses to mutant proteins also varied. V169fs, R185fs, and S226fs mutants induced the expression of ER stress markers, while L184fs mutant did not alter the level of BiP or CHOP (Fig. 1B). To further investigate the effect of wild type and mutant PMP22 mediated ER stress, cell viability was measured after transfection. MTT assay results revealed cell death was induced by both forms of PMP22 in a dose dependent manner (Fig. 1C).

Immunocytochemical analysis was used to confirm whether the changes of ER stress markers are related to ER retention. Both mutant proteins, PMP22-L16P and MPZ-R185fs, colocalized with PDI, an ER resident protein, in S16 cells (Fig. 2). Therefore, these data imply that expression of PMP22 and
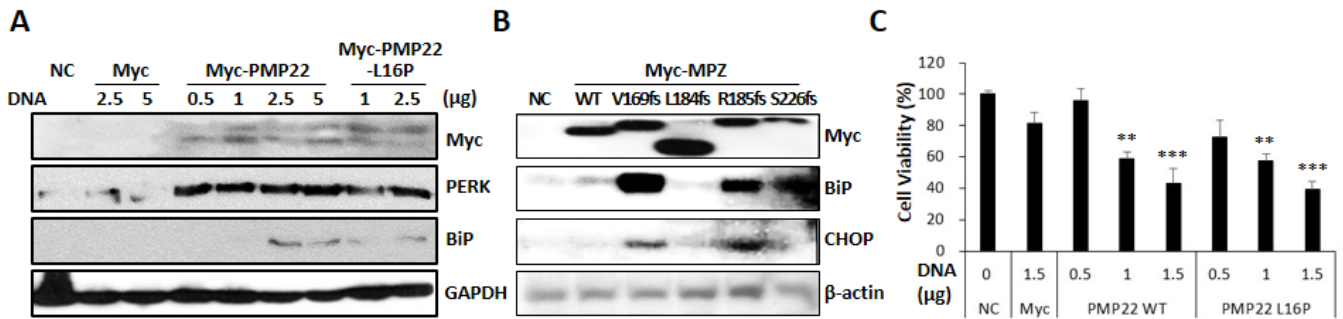
Fig. 1. Elevation of endoplasmic reticulum (ER) stress marker levels by expression of peripheral myelin protein 22 (PMP22) and myelin protein zero (MPZ) proteins. (A) ER stress markers are induced by wild type and mutant PMP22 in a dose dependent manner. The indicated amount of DNA was transfected into HEK293 cells for 48 hours. (B) Mutant MPZ protein expression induced ER stress markers in HEK293 cells. (C) Expression of wild type and L16P mutant PMP22 resulted in cell death in HEK293 cells. Cell viability was determined by the MTT assay as described in the Materials and Methods section. Three independent experiments were performed and statistical significance was compared with Myc-control DNA using the Student's t-test. Data are presented as mean±standard error of mean. **$P<0.01$, ***$P<0.001$. NC, no transfection control.
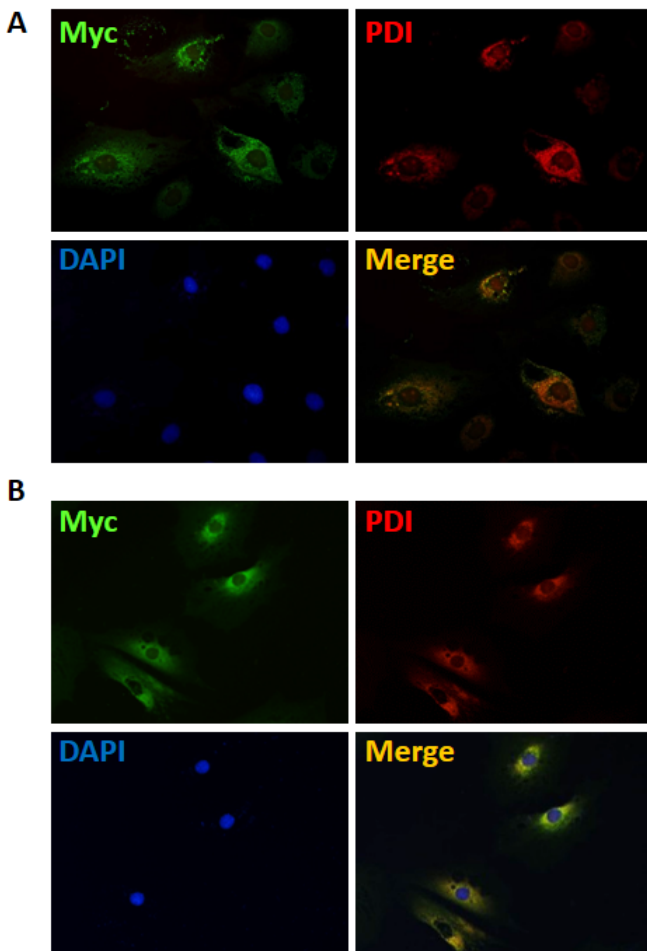

Fig. 2. Localization of mutant peripheral myelin protein 22 (PMP22) and myelin protein zero (MPZ) proteins. Myc-tagged PMP22-L16P (A) and MPZ-R185fs (B) DNA was transfected into S16 cells for 24 hours. Localization of each protein was determined using immunocytochemistry. Anti-protein disulfide isomerase (PDI) antibody and 4′,6-diamidino-2-phenylindole (DAPI) were used to indicate endoplasmic reticulum and nuclear localization, respectively.

MPZ mutant forms causes ER retention resulting in ER stress in Schwann cells.

2. Generation of chemically induced ER stress models

Since accumulation of mutant PMP22 and MPZ proteins leads to ER stress, we next sought to establish a more stable cell model for further development of high-throughput screening (HTS) by mimicking genetic models. Several chemicals are reported to induce ER stress; however, none have been demonstrated in Schwann cells.

To generate a chemically induced ER stress model in Schwann cells, we treated cells with CI, BFA, TM, or TG for 24 hours. CI treatment did not induce effective ER stress-mediated cell death in either primary Schwann cells or S16 cells (data not shown). However, treatment of TG (Fig. 3A), TM (Fig. 3B), and BFA (data not shown) induced cell death in a dose dependent manner in both cell types. Overall, primary Schwann cells were more vulnerable to ER stress inducers.

To determine whether reduced cell viability is correlated with ER stress responses, we measured the changes of CHOP, an ER stress-associated apoptotic protein, after treatment with TG. Treatment with TG for 6 hours was sufficient to induce the expression of ER stress markers in both primary Schwann cells and S16 cells (Fig. 3C). These data demonstrated that TG, or other chemicals, can induce ER stress similar to mutant PMP22 and MPZ proteins in Schwann cells.

Discussion

There are currently no treatment options for CMT. Recently, vitamin C application showed promising therapeutic efficacy in animal models [19]. However, it has not been successful in
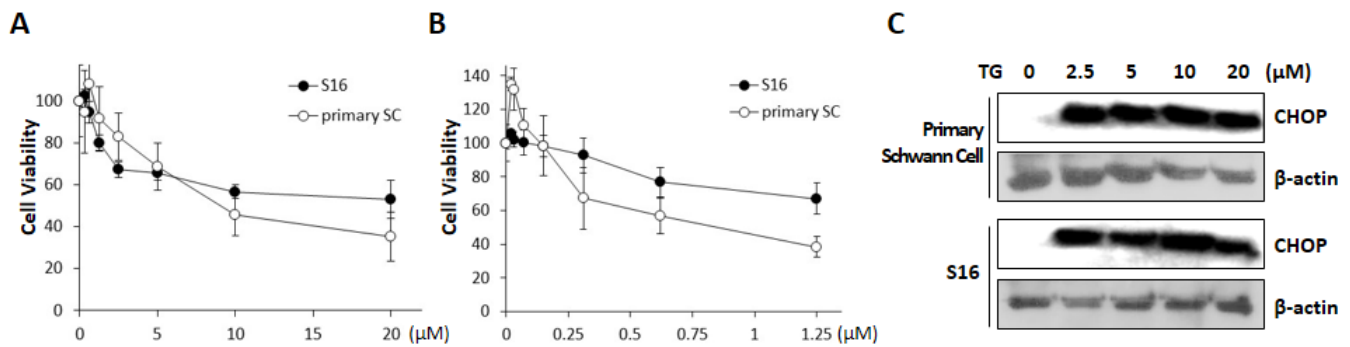
Fig. 3. ER stress-mediated cell death. The indicated concentrations of TG (A) and TM (B) were applied to primary Schwann cells and S16 cells for 24 hours. Cell viability was determined by the MTT assay. Data are representative of three independent experiments. (C) Chemically induced ER stress in both primary Schwann cells and S16 cells was determined after treatment of the indicated concentrations of TG for 24 hours. Expression levels of the ER stress marker CHOP were measured. ER, endoplasmic reticulum; TG, thapsigargin; TM, tunicamycin; SC, Schwann cell.

clinical trials [20]. Several other therapeutic strategies effective in animal models have not been clinically tried yet [21,22]. To assist in the development of new therapeutics, a HTS system was developed to search for PMP22 transcriptional modulators capable of addressing the gene dosage problem, which is related to duplication of the gene [23]. Using a reporter system, they screened over 3,000 chemicals and found several transcriptional modulating candidates.

Twenty *PMP22* and 36 *MPZ* mutations have been listed in OMIM (Online Mendelian Inheritance in Man). Among them, several mutations have been well characterized to induce ER stress [14,17]. Structurally, ER retention mechanisms by the PMP22 L16P mutant are well explained. Induction of ER stress markers caused by MPZ mutations were also characterized. Mutant MPZ proteins are also retained in the ER and trigger unfolded protein responses (UPR) that are measured by the UPR markers BiP, CHOP, and genes downstream of CHOP [24].

To combat ER stress caused by PMP22 and MPZ mutations, oral administration of curcumin was tested in animal models and exhibited improvement of ER protein retention; in addition, it reduced peripheral neuropathy severity in both CMT1A and CMT1B mouse models [17,25]. Thus targeting ER stress might be a promising treatment option for CMT1. In this context, generation of HTS systems might be necessary for future drug development. In this study, we chose several PMP22 and MPZ mutants: L16P mutation in PMP22 and V169fs, L184fs, R185fs, S226fs mutations in MPZ. They have been well characterized in previous reports and occur in high frequencies within the genes.

In this study, we used both genetic and chemical induction systems. Transient expression of mutant proteins was enough to trigger ER stress and cell death. However, this model lacks critical options for further drug discovery. For instance, the genetically induced cells could not be used for stable cell lines to generate a reproducible system due to viability. Therefore, we explored another system to substitute the genetic model. Several chemicals, such as TG, can trigger ER stress by mimicking genetic stress [26]. Our data revealed that TG induces ER stress-mediated cell death in Schwann cells, which is the main pathological mechanism in CMT1. Therefore, chemically induced ER stress models can stably substitute mutant proteins causing ER stress.

In conclusion, we have developed a chemically induced ER stress model using Schwann cells. Application of this model to a HTS system might facilitate the development of therapeutic options for CMT1.

Acknowledgements

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