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Oral Curcumin Mitigates the Clinical and Neuropathologic Phenotype of the *Trembler-J* Mouse: A Potential Therapy for Inherited Neuropathy

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Mutations in myelin genes cause inherited peripheral neuropathies that range in severity from adult-onset Charcot-Marie-Tooth disease type 1 to childhood-onset Dejerine-Sottas neuropathy and congenital hypomyelinating neuropathy. Many myelin gene mutants that cause severe disease, such as those in the myelin protein zero gene (*MPZ*) and the peripheral myelin protein 22 gene (*PMP22*), appear to make aberrant proteins that accumulate primarily within the endoplasmic reticulum (ER), resulting in Schwann cell death by apoptosis and, subsequently, peripheral neuropathy. We previously showed that curcumin supplementation could abrogate ER retention and aggregation-induced apoptosis associated with neuropathy-causing *MPZ* mutants. We now show reduced apoptosis after curcumin treatment of cells in tissue culture that express *PMP22* mutants. Furthermore, we demonstrate that oral administration of curcumin partially mitigates the severe neuropathy phenotype of the *Trembler-J* mouse model in a dose-dependent manner. Administration of curcumin significantly decreases the percentage of apoptotic Schwann cells and results in increased number and size of myelinated axons in sciatic nerves, leading to improved motor performance. Our findings indicate that curcumin treatment is sufficient to relieve the toxic effect of mutant aggregation-induced apoptosis and improves the neuropathologic phenotype in an animal model of human neuropathy, suggesting a potential therapeutic role in selected forms of inherited peripheral neuropathies.

Human inherited peripheral neuropathies are often a manifestation of peripheral myelin dysfunction and have long been proposed to result from abnormalities in Schwann cells and their myelin sheath and perturbed axon-glia interactions. The hereditary motor and sensory neuropathies are the largest class of hereditary neuropathies and include Charcot-Marie-Tooth disease (CMT) types 1 and 2 (CMT1 [MIM 118200] and CMT2 [MIM 600882]), hereditary neuropathy with liability to pressure palsies (HNPP [MIM 162500]), Dejerine-Sottas neuropathy (DSN [MIM 145900]), and congenital hypomyelinating neuropathy (CHN [MIM 605253]). CMT is the most common inherited disorder of the peripheral nervous system, with an estimated frequency of 1 in 2,500 individuals.[1] Patients with CMT usually manifest symptoms in the 1st or 2nd decade, with slowly progressive, symmetrical, length-dependent neuropathy leading to weakness of the distal muscles of the legs and feet, followed in most cases by involvement of the hands.[2–4] CMT1, the demyelinating form, is characterized by a slowing of the motor-nerve conduction velocities (usually to <38 m/s).[2]

The molecular cause of CMT1 in the majority of patients is a duplication of a 1.4-Mb region on the short arm of chromosome 17, to which the dosage-sensitive peripheral myelin protein 22 gene (*PMP22*) maps.[5–7] The reciprocal deletion of the same region is associated with a milder disease, HNPP.[8–10] PMP22, a 22-kDa glycoprotein with four putative transmembrane domains, comprises 2%–5% of peripheral nervous system myelin[11] and is found uniformly in compact regions of myelin.[12,13] Although PMP22 is widely expressed, by far the largest amount of PMP22 is produced by myelinating Schwann cells. It is clear from both human studies[7] and animal models[14] that alteration in *PMP22* gene dosage and expression has profound effects on the development and maintenance of peripheral nerves.[2,10] Consequently, the regulation of *PMP22* gene expression has been the focus of therapeutic strategies for CMT1A.[15–16] Onapristone, a progesterone antagonist, slows the disease progression by reducing the level of overexpression of *PMP22* in a CMT1A transgenic rat model, consequently improving the CMT phenotype.[15] Ascorbic acid treatment also significantly improves locomotor performance of CMT1A transgenic mouse models, possibly by blunting stimulation of cyclic adenosine monophosphate and thereby reducing the expression of *PMP22* to below the threshold level, enough to partially ameliorate the neuropathy.[16] These therapeutic approaches show promise in animal studies, but they are not feasible for other genetic causes of CMT because such molecular strategies apply only to *PMP22* overexpression.

PMP22 point mutations can also cause demyelinating neuropathies, such as CMT1, DSN, CHN, and HNPP (In-

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herited Peripheral Neuropathies Mutation Database). The spontaneously occurring mouse models for CMT1, *Trembler-J* (*Tr-J*) (with mutation L16P) and *Trembler* (*Tr*) (with mutation G150D) result from dominantly transmitted *PMP22* missense mutations.[17,18] More recently, additional mouse *Pmp22* mutations that phenocopy more-severe human peripheral neuropathies, such as DSN, were isolated in a large-scale ethylnitrosourea mutagenesis screen.[19] Of note, severely affected patients with CMT1 with identical mutations and nerve biopsy results showing alterations analogous to those detected in heterozygous *Tr-J* and *Tr* mice have been described.[14,20,21] The pathomechanism for how these mutations affect myelination remains unknown; however, recent studies have shown that post-translational events, such as protein processing, trafficking, and accumulation in different intracellular compartments, are critical for myelination of Schwann cells.[22–25]

Recent experiments in diverse disease animal models suggest that curcumin enables misfolded proteins to traverse from the endoplasmic reticulum (ER) to the plasma membrane,[26–29] concurrently reducing the cytotoxicity of the mutant protein. Such observations have led us to explore the effects of curcumin in wild-type *PMP22, Tr-J,* and *Tr* mutants, using both cell-based and whole-animal assays. We studied the effects of curcumin on the wild-type and aggregation-induced apoptosis associated with disease-causing mutant PMP22 in transiently transfected HeLa cells. As we had observed previously for disease-associated *MPZ* alleles,[29] the Tr-J and Tr mutant proteins appear to be partially released from the ER after curcumin treatment; this is also associated with reduced apoptosis of cells in tissue culture. To evaluate the effects of curcumin in vivo, curcumin was administered orally to *Tr-J* mice. We observed considerable improvement of the neuropathy, including both the motor performance and the histopathology of the treated *Tr-J* mice, suggesting a potential therapeutic use for curcumin in severe forms of inherited peripheral neuropathy.

Material and Methods

Recombinant Constructs

Full-length human *PMP22* cDNA was subcloned into expression vector pcDNA3.1 (Invitrogen). Mutations were generated in each construct by use of the QuikChange site-directed mutagenesis kit (Stratagene). Primer sets were designed to create the following mutations: 47T→C (L16P; *Tr-J*) and 449G→C (G150D; *Tr*). Clones were verified by direct double-stranded DNA sequencing with use of the DyePrimer chemistry and ABI 377 sequencer (Applied Biosystems).

Tissue Culture, Transfection, and Immunostaining

HeLa cells were grown on a 6-well chamber flask and 4-well slides in Dulbecco's modified Eagle medium (BioWhittaker), supplemented with 10% fetal bovine serum, and were transfected with use of Lipofectamine 2000 (Invitrogen) in accordance with the supplier's instructions. Cells were incubated for 24 h at 37°C in a humidified incubator containing 10% CO_2. Cells were fixed with 2% paraformaldehyde in PBS at room temperature for 10 min. The fixed cells were then washed and permeabilized with 0.1% Triton-X 100 in PBS on ice for 2 min. The cells were rinsed twice with PBS and were blocked with 5% normal goat serum in PBS for 1 h at 37°C. They were incubated with primary antibodies diluted in PBS with 1% normal goat serum at appropriate concentrations for 1 h at 37°C. The primary antibodies used in this study included the rabbit polyclonal antibody against PMP22 protein (1:1,000 [Novus Biologicals]) and the mouse monoclonal protein disulfide isomerase (PDI) (1:2,000 [Affinity Bioreagents]). This incubation was followed by two washes in PBS and incubation with Alexa Fluor goat anti-rabbit antibody (1:1,000 [Invitrogen Molecular Probe]) for 1 h at 37°C. For visualization of the nuclei, SlowFade Antifade Kit with 4′6-diamino-2-phenylindole (DAPI) (Invitrogen Molecular Probe) was used in accordance with the manufacturer's instructions. Fluorescently labeled cells were visualized by standard fluorescent microscopy.

Apoptosis Assay and Flow-Cytometric Analysis

Terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) staining was performed with use of an in situ cell-death detection kit, Fluorescein (Roche Applied Science). Cells were grown in four chamber slides and were fixed with 4% paraformaldehyde in PBS at room temperature for 10 min. Cells were then washed and permeabilized with 0.1% Triton-X 100 in PBS on ice for 2 min. TUNEL staining was performed in accordance with the conditions recommended by the supplier (for 1 h at 37°C). The average numbers of TUNEL-positive and DAPI-positive cells were calculated in 10 different sections, and the SD of the ratio was determined for each slide. Student's *t* tests comparing wild-type PMP22 and its mutants were performed. Statistical significance was defined by $P < .05$. For fluorescence-activated cell sorting (FACS) analysis, cells were transiently transfected for 24 h and then were harvested. Staining with annexin V, fluorescein isothiocyanate (FITC), and propidium iodide (BD Biosciences Pharmingen) was performed by the incubation of cells (1×10^6 cells/ml) in the dark for 25 min at room temperature in a binding buffer (10 mM HEPES, 140 mM NaCl, and 2.5 mM $CaCl_2$, at pH 7.4) containing a saturating concentration of annexin V, FITC, and propidium iodide. After incubation, the cells were washed, pelleted, and analyzed in an FACScan analyzer (Becton Dickinson). For in vivo TUNEL analysis, sciatic nerves were removed from mice immediately after death and were cut and fixed in 2.5% gluteraldehyde and 0.5% paraformaldehyde in 0.1 M phosphate buffer for at least 2 h. Nerve segments were then dehydrated in a graded series of ethanol and were embedded in paraffin. Longitudinal sections, 7 μm thick, were mounted on slides and then were dewaxed and rehydrated. In vivo TUNEL analysis was performed using ApopTag Peroxidase *In Situ* Apoptosis Detection kit (Chemicon International) in accordance with the supplier's instructions.

Genotyping

Genomic DNA was isolated from the tails of newborn mouse pups, and *Tr-J* mice (C57BL/6J) were genotyped as described elsewhere.[22]

Curcumin Treatment

Curcumin was purchased from Sigma (catalog number C7727). For cell culture treatment, curcumin stock was dissolved in di-