Mutant Cu/Zn superoxide dismutase deposition and dismutation kinetics in cell culture

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Amyotrophic Lateral Sclerosis (ALS)

is a common neuromuscular disease without effective treatment characterized by the selective degeneration of motor neurons resulting in paralytic death. In a familial subset of ALS, mutant Cu/Zn superoxide dismutase (**SOD1**) enzymes initiate and sustain this disorder dose-dependently. SOD1 is a highly conserved 153-residue β-barrel homodimer that serves as the predominant intracellular scavenger of neurotoxic superoxide anion radical ($O_2^{\cdot-}$). Determining the gain-of-function mechanism of SOD1 toxicity for therapeutic intervention in ALS has been the subject of continuing research for over fifteen years.

Pathogenic SOD1 species aberrantly oligomerize,

and a portion of these multimers form cytoplasmic mass aggregates. Aggregate depositions are primarily detected biochemically and histologically, the data of which has led to several implicate observations:

- Every toxic SOD1 mutant assayed thus far forms distinct but relatively variable detergent-insoluble aggregates whereas wildtype (**WT**) and other innocuous SOD1s remain persistently soluble (1, Fig.1).
- Relative to a mutant standard, mutant aggregation potential in HEK cell culture is correlate with a shorter disease duration in patient cohorts (1).
- Spinal cord extracts of symptomatic transgenic mice show rapid accumulation of aggregate SOD1 over the course of disease (2), and deposition occurs near-selectively in the ventral horn (3).

Mutant SOD1 dossiers: D101N and D101G

Historically, mutant comparisons have been helpful for delineating toxic function. For examples: the verified metal-free (consequently, inactive or scavenging-free) double Cu-binding histidine knockout H46R/H48Q is still paralytic and aggregate-prone, as is a mutant mouse SOD1 with a glycine lesion homological to human G85R. This study investigates mutant isoforms at aspartic acid residue 101. D101N is a relatively common ALS mutant, but with unique WT-like properties: its metallation status, H/D exchange, DSC profile, UV and EPR spectroscopy data match that of WT (4). D101N does not aggregate at 24h in HEK cell culture, whereas D101G does so robustly seemingly as soon as protein is synthesized. Patients carrying D101N and D101G are clinically identical with average ages of onset of 41.0 ± 10 and 48.0 ± 9.1 years and survival times of 2.4 ± 0.9 and 2.5 ± 0.4 years, respectively (N=14 and 3, 1).

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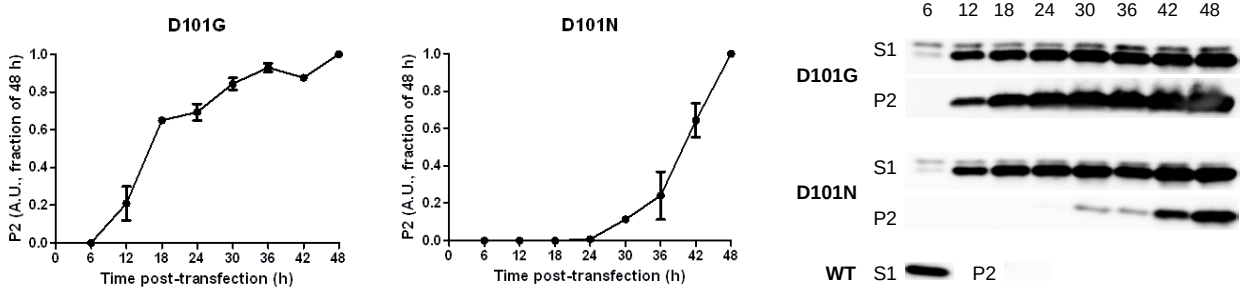

Figure 1. Sequential timed detergent extractions. D101N and D101G deposition levels in transfected HEK cells as assayed by detergent/ultracentrifugation extraction (detailed in materials and methods). D101G aggregates appear promptly and abundantly whereas D101N is considerably slower to nucleate. WT does not form detergent-insoluble aggregates at any time.

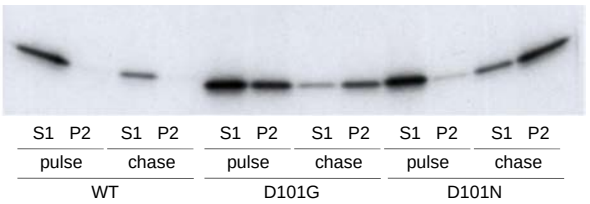

Figure 2. ^{35}S-cysteine pulse-chase detergent extractions. 24 h after HEK cell transfection, samples were radiolabeled for 1 h and harvested immediately ("pulse") or after growing 24 h in *cold* medium ("chase"). Aggregates at 49 h post-transfection are composed of proteins that were predominantly soluble 24 h before for both D101N and D101G, but not WT.

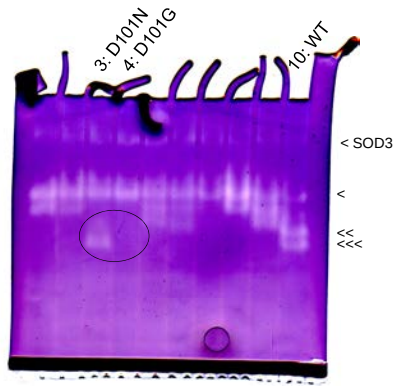

Figure 3. Native nitro blue tetrazolium superoxide scavenging gel assay. D101N, D101G, and WT among other mutant SOD1s in 3T3s. D101G loses dismutase activity, whereas D101N appears WT-like except with decreased mouse heterodimer formation. Extracellular Mn-SOD (SOD3) is noted, as are homodimeric endogenous mouse SOD1 (<), mouse/human SOD1 heterodimer (<<), and human SOD1 homodimer (<<<) on the basis of electrophoretic mobility.

MATERIALS AND METHODS

Cell culture and transfections. SOD1 cDNAs coded in the mammalian expression vector pEF-BOS were prepared by double CsCl/EtBr density gradient and confirmed by automated sequencing and agarose electrophoresis. 4 μg cDNA transfectant was prepared with Lipofectamine 2000 (Invitrogen) and added to confluent 60mm dishes of human embryonic kidney cells (HEK cells, line HEK293FT) or mouse embryonic fibroblasts (3T3s, line NIH 3T3). Cells were passaged in high-glucose DMEM with 10% serum (horse for HEK cells, newborn calf for 3T3s), supplemented with L-glutamine. Fresh media was added 4h after transfection, and cell pellets were harvested to -80˚C for storage after rinsing three times in phosphate-buffered saline (PBS).

SOD1 aggregate extraction and immunoblotting. Cell pellets were thawed on ice and resuspended in 100 μL 1x TEN (10 mM Tris pH 7.4, 1 mM EDTA pH 8.0, 100 mM NaCl). 100 μL 1x TEN with 1% Nonidet P40 (NP-40) and 1% protease inhibitor cocktail (PI, Roche) was then added for a final concentration of 0.5% NP-40. This mixture was then sonicated and centrifuged for 5 min at >100,000 x *g* in a Beckman Airfuge. The soluble portion was saved as *S1* and the pellet resuspended in 200 μL of rinse buffer: 1x TEN with 0.5% NP-40 and 1% PI. The pellet was sonicated and centrifuged again, this time discarding the soluble fraction. The pellet was finally resuspended and resonicated in 1x TEN with 0.5% NP-40, 0.25% SDS, 0.5% deoxycholate, and 1% PI and saved as *P2*. Protein concentrations were assayed by the BCA method (BSA standard). 5 μg S1 or 20 μg P2 were brought up to 20 μL in 1x TEN with laemmli buffer (5% β-mercaptoethanol). Samples were boiled at 95 ˚C for 6 min and loaded on 18% tris-glycine acrylamide gels. Gels were transferred to nitrocellulose membrane for 2 h at 400 mA and then blocked for 15-40 mins in 5% lowfat dry milk in PBS-T (PBS with 0.1% Tween-20). Primary anti-m/hSOD1 antibody was added 1:5000 in milk from PBS-T for 1-16 h followed by 3x rinse in PBS-T alone and secondary goat anti-rabbit HRP at 1:2500 in milk from PBS-T for 1 h. Blots were rinsed again and visualized and pixel-quantified with ECL chemiluminescence on a Fujifilm LAS-3000.

^{35}S-cysteine pulse-chase analysis. 24 h after transfection, HEK cells were pulse radiolabeled with ^{35}S-cysteine for one hour. Some samples were harvested immediately, other samples were chased with cold media for 24 h before harvesting for detergent extraction and immunoprecipitation with anti-m/hSOD1, followed by SDS-PAGE and autoradiography.

Superoxide anion scavenging activity assay. < 100 μg total cell lysate prepared from 10x pellet volume sonicated 0.1% NP-40 in 1x TN was run on 8% or 4-20% tri-glycine acrylamide gels in 1x TG with 20% methanol at 4˚C and 100 constant V for 4-6 h, without denaturant or reductant added. The gel was retrieved and soaked in 50 mM potassium bicarbonate buffer containing 65 μg/mL riboflavin and 280 μg/mL nitro blue tetrazolium, pH 7.6 (5). After incubating 2-40 m, the solution was aspirated and 0.1% TEMED in 50 mM potassium bicarbonate buffer was added to the gel. The gel was immediately exposed to white light from a bright box and imaged on a Agfa Duoscan, with contrast increased.

SUMMARY AND CONCLUSIONS

- Both D101G and D101N form significant detergent-insoluble aggregates in HEK cell culture, although D101N nucleation is relatively delayed.
- Nonetheless D101N also has a high deposition efficiency. The majority of soluble radiolabeled proteins at 24 h after transfection are ultimately converted to the insoluble conformer for both D101N and D101G.
- The D101G lesion results in a loss of dismutase activity, whereas the D101N mutant is electrophoretically and enzymatically WT-like.

Subspecific immature mutant proteins lacking metals and an intrasubunit disulfide bond likely nucleate the oligomerization pathway ultimately to insoluble aggregates. Since the mutations studied share a residue, one would not expect significant structural variation between D101N and D101G oligomers. Apparent by its loss of enzymatic activity, D101G may supply a larger pool of immature protein than D101N. More mechanistic work is prudent for attempts at clinical applications.