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 153-residue β-barrel homodimer that serves as the predominant cytosolic 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 mito/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 published thus far forms distinct but relatively variable detergent-insoluble aggregates whereas wildtype (**WT**) and other innocuous SOD1s remain persistently soluble (1 and references therein).
- 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).

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). Cells were maintained in high-glucose DMEM with 10% horse serum 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 96 ˚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 15-240 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. **Semi-native analysis.** Equivalent protein from S1s, P2s, or raw 1x TN freeze thaw (F/T) extracts were loaded with ~20% glycerol and minimal dye on 4-20% 1x TG gels without SDS and run at 100 constant volts at 4˚C for 4-8 hours. Gels were retrieved. Some gels were treated with 2% SDS 2% βME 1x TG and microwaved for pulses of 20 seconds each, 2 minutes total for in-gel reduction. Native gels were transferred to nitrocellulose and immunolabeled as described above.

REFERENCES

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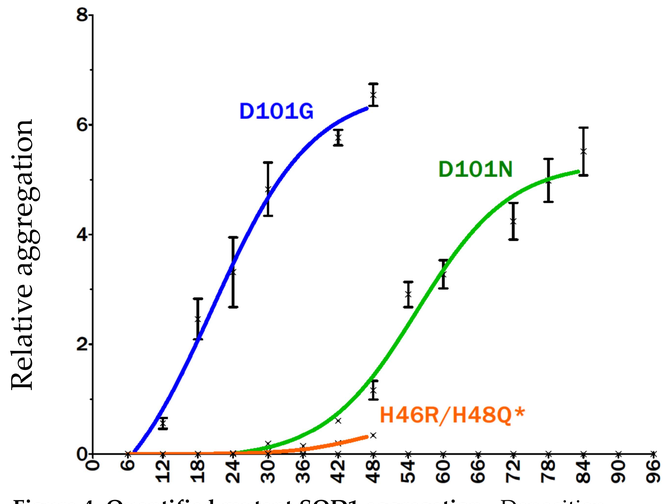


Figure 4. Quantified mutant SOD1 aggregation. Deposition levels in transfected HEK cells as assayed by detergent/ultracentrifugation extraction (detailed in materials and methods). Immunolabeled mutant protein at the apparently reduced monomeric position was pixel-quantified, with 24h A4V set to 1. D101G aggregates appear promptly and abundantly whereas D101N is considerably slower to nucleate. Wildtype, familial mutant E133V, and two of three artificial* mutants do not form detergent-insoluble aggregates at any time. N=2-4.

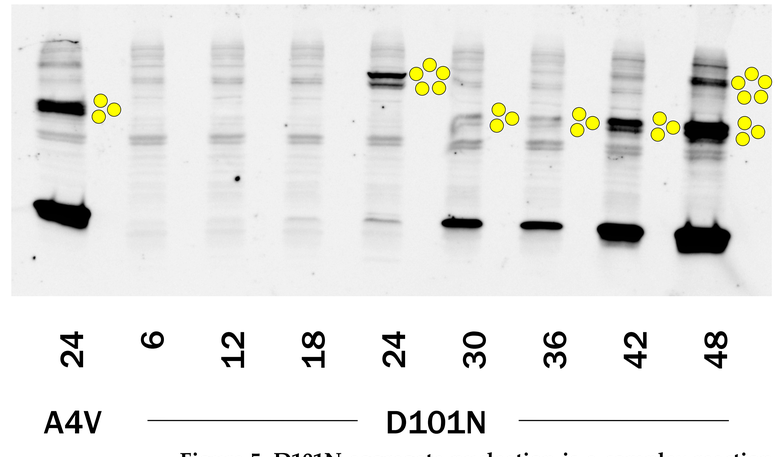
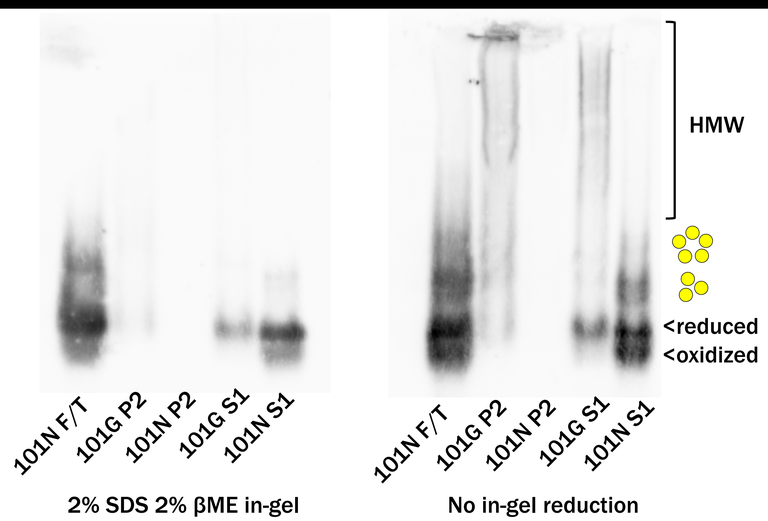


Figure 5. D101N aggregate nucleation is a complex reaction involving oligomeric intermediates or other SOD1-reactive adducts. Apparent trimers and apparent pentamers (each with two isoforms, between 40-60 kDa and at 80 kDa respectively, based solely on post-stained molecular weight markers) are noted. Preemptive 24h apparent pentamer N=2, all else N=3. D101N expression stabilizes between 12 and 18 hours. There is virtually no expression at 6 hours post-transfection, and the 6 hour lane shown is representative of high-exposure background in control P2s.



Harvest 42h post-transfection

Figure 6. Semi-native immunoblotting. Analysis of soluble and insoluble mutant SOD1 in transfected HEK cells as assayed by detergent/ultracentrifugation extraction (detailed in materials and methods). Intrareduced and intraoxidized protein is noted, as are apparent trimers/pentamers and high molecular weight species, soluble and not.

CONCLUSIONS

Both D101N and D101G form significant detergent-insoluble aggregates in HEK cell culture, although D101N nucleation is delayed. D101N&G carriers have just 2.5 years average time from diagnosis to death. D101N&G have similar rates of deposition and a similar threshold but dissimilar nucleation, indicating metrics that may be more correlative with clinical data. This is not the case for all variable mutants at one position. More time course and pulse chase experiments are underway to test this hypothesis.

The D101G lesion results in a loss of dismutase activity (and intraoxidized protein), whereas the D101N mutant is electrophoretically and enzymatically WT-like. Notably, the apparent trimers and pentamers are resistant to 5% βME and appear in both the S1 and P2 fractions, as do E133V's βME-resistant oxidized disulfides. E133V retains no aggregate intrareduced protein even in extended cell culture. The E133V::YFP fusion protein is comparable to WT::YFP in that neither aggregate in adherent cells even in extended cell culture.

SOD1s extracted in the presence of NP-40 and EDTA have diminished activity and tightened bands relative to TN extracts (data not shown). Thus S1 assays are an underrepresentation of actual activity. However, it does not appear the degree to which detergents or chelators effect SOD1 proteins vary significantly interspecifically. Although it is unlikely insoluble SOD1s are active, the detergent extract design (rinses and SDS/deoxycholate) is not suitable for enzymatic analysis.