Aggregation and Motor Neuron Toxicity of an ALS-Linked SOD1 Mutant Independent from Wild-Type SOD1


Analysis of transgenic mice expressing familial amyotrophic lateral sclerosis (ALS)–linked mutations in the enzyme superoxide dismutase (SOD1) have shown that motor neuron death arises from a mutant–mediated toxic property or properties. In testing the disease mechanism, both elimination and elevation of wild-type SOD1 were found to have no effect on mutant–mediated disease, which demonstrates that the use of SOD mimetics is unlikely to be an effective therapy and raises the question of whether toxicity arises from superoxide-mediated oxidative stress. Aggregates containing SOD1 were common to disease caused by different mutants, implying that coaggregation of an unidentified essential component or components or aberrant catalysis by misfolded mutants underlies a portion of mutant–mediated toxicity.

After the discovery (1) that dominant missense mutations in Cu/Zn superoxide dismutase (SOD1) were the primary cause of 15 to 20% of familial ALS cases, experiments with transgenic mice expressing ALS-linked mutations showed that selective motor neuron degeneration arises from toxic properties of the ubiquitously expressed mutant enzymes (2–5). This has fueled proposals that (i) mutant SOD1s have an altered substrate affinity that leads to high levels of toxic reaction products (6–8); (ii) the reduced SOD activity arising from unstable or imperfectly folded enzymes causes toxic oxidative stress through an imbalance in oxidative defenses (9, 10), which in turn may exacerbate aberrant catalysis; or (iii) poorly or unstably folded mutants mitigate SOD1-containing aggregates that are toxic to motor neurons (5, 11).

To test these hypotheses, the influence of wild-type SOD1 polypeptide and activity levels on the onset and progression of SOD1 mutant toxicity was determined by eliminating or elevating wild-type SOD1 in mice developing motor neuron disease due to expression of an ALS-linked mutant with glycine substituted to arginine at position 85 (SOD1G85R). Because in vivo the SOD1G85R mutant polypeptide appears to retain only about 10% of the activity of the wild-type SOD1 (at least when expressed in yeast) (12) and disease arises with low levels of the mutant protein (5), neither SOD1 activity nor polypeptide levels are markedly elevated in the SOD1G85R animals.

SOD1G85R mice were mated to mice expressing wild-type human SOD1 at six times the level of endogenous mouse SOD1 (13). In addition, a two-stage breeding strategy (13) was used with mice carrying a targeted deletion of the endogenous SOD1 gene (14) to generate mice expressing SOD1G85R in the absence of endogenous SOD1, along with littermates with one or both endogenous SOD1 alleles. Total spinal cord extracts from two animals from each genotype were immunoblotted with an antipeptide antibody (15) that reacts exclusively with human, but not mouse, SOD1. Neither a sixfold increase in wild-type SOD1 (Fig. 1A) nor its complete elimination (Fig. 1B) affected the accumulated levels of mutant SOD1G85R protein. Thus, despite a decreased stability of SOD1G85R relative to wild-type SOD1 in these mice (5), the wild-type protein did not stabilize mutant SOD1. Moreover, the presence of SOD1G85R had no effect on the level (Fig. 1A) or the activity of wild-type SOD1.

Survival plots comparing transgenic mice expressing both SOD1G85R and wild-type human SOD1 (Fig. 2A, solid squares, n = 20) or SOD1G85R alone (Fig. 2A, open squares, n = 14) indicated that increasing wild-type SOD1 activity and polypeptide level had no beneficial effect. Age distributions at end-stage disease were indistinguishable (with the exception that a small fraction of mice with normal SOD1 levels survived up to 20 days longer). There were no differences in age of disease onset, and duration after onset was also constant (a median of 18 days for both genotypes). Similarly, no differences in age at end-stage disease could be seen among mice expressing SOD1G85R in the presence of normal levels of mouse SOD1 (Fig. 2B, triangles, n = 5) when levels of mouse SOD1 were reduced to 50% of normal levels (Fig. 2B, open circles, n = 10) or in the complete absence of endogenous mouse SOD1 (Fig. 2B, solid circles, n = 5).

A prominent pathology found in all mouse lines that develop ALS-like disease from expressing SOD1G85R is neuronal and astrocytic inclusions that appear in the SOD1G85R mice before clinical signs, increase markedly

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**Fig. 1.** The presence or absence of wild-type protein does not affect the stability of mutant protein. (A) Levels of wild-type human SOD1 or mutant SOD1G85R determined by immunoblotting spinal cord extracts from normal mice (control), transgenic mice expressing wild-type human SOD1 to six times the level of endogenous mouse SOD1 (hSOD1), transgenic mice expressing SOD1G85R (G85R), or mice carrying both the hSOD1 and SOD1G85R transgenes (G85R, hSOD1). Each pair of slots represents analyses from duplicate animals. Human SOD1 was detected with a peptide antibody specific for human, but not mouse, SOD1 (5). (B) Total spinal cord extracts from mice transgenic for SOD1G85R and carrying both endogenous SOD1 alleles (G85R, SOD1G85R) or carrying a deletion of one (G85R, SOD1−/−) or both (G85R, SOD1−/−) endogenous SOD1 alleles. Top panel: Extract proteins detected with Coomassie blue; middle and bottom panels, immunoblots with antibodies specific for human SOD1 (middle) or mouse SOD1 (bottom) (5, 25).
in abundance during disease progression (5), and are highly immunoreactive for SOD1 that is concentrated either at the periphery of the inclusion or throughout it (Fig. 3B, arrowheads) or more diffusely localized in aggregates (Fig. 3B, arrows), many of which (for example, Fig. 3B, lower right arrow) are not identified by conventional histological stains (Fig. 3A). Examination of mice expressing other SOD1 mutants revealed that this is a common pathology of SOD1 mutant–mediated ALS in mice, because diffuse aggregates that are strongly immunoreactive for SOD1 are found in both astrocytes and neurons of lines expressing high levels of SOD1G93A (Fig. 3C), as well as one line expressing low levels of SOD1GS37R (16) and in neurons in several SOD1G37R lines (17). Neither type of SOD1 aggregate was found at any age in mice expressing wild-type human SOD1. Similar SOD1-containing aggregates have also been reported for some human sporadic (18–20) and familial (21–24) ALS cases, including a familial ALS patient with a 2-base pair deletion at codon 126 of SOD1 (Fig. 3, E and F). Because this latter mutation truncates the final 27 amino acids of SOD1 (23), strong immunostaining of these aggregates with an antipeptide antibody against SOD1 amino acids 125 through 137 (25) showed that wild-type SOD1 is an apparently prominent component of these aggregates (Fig. 3F).

To examine whether wild-type SOD1 exacerbated the aggregates, pathology arising during disease progression was determined in SOD1G85R mice with varying levels of wild-type SOD1. Before the onset of symptoms (at ages up to 7.5 months), there were no SOD1-positive inclusions or reactive astrocytes in spinal cords from the any of the mice and no axonal degeneration in dorsal or ventral roots. A 70% loss of large (>5 μm) caliber motor axons in the L5 ventral root was seen at end-stage disease in SOD1G85R transgenic mice independent of the wild-type SOD1 content, whereas the small-caliber axons were almost completely spared (Table 1). Similarly, in all genotypes the numbers of large surviving axons in G85R, G85R, SOD1−/−; G85R, SOD1−/+; and G85R, hSOD1 mice. For all genotypes, three mice were analyzed, except that marked with an asterisk, for which two animals were measured.

Table 1. Surviving large and small motor axons at end-stage disease in the L5 ventral roots of mice with normal, reduced, or elevated levels of wild-type SOD1. Statistical tests (analysis of variance, P = 0.826) revealed no significant difference between the numbers of large surviving axons in G85R; G85R, SOD1−/−; and G85R, hSOD1 mice. For all genotypes, three mice were analyzed, except that marked with an asterisk, for which two animals were measured.

<table>
<thead>
<tr>
<th>Survival axes</th>
<th>Wild-type mice (12 months)</th>
<th>G85R</th>
<th>G85R, SOD1−/−</th>
<th>G85R, SOD1−/+</th>
<th>G85R, hSOD1</th>
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<tr>
<td>Large (&gt;5 μm)</td>
<td>730 ± 12</td>
<td>188 ± 13</td>
<td>145 ± 51</td>
<td>221 ± 138</td>
<td>183 ± 18</td>
</tr>
<tr>
<td>Small (=5 μm)</td>
<td>304 ± 23</td>
<td>295 ± 24</td>
<td>293 ± 5</td>
<td>234 ± 2</td>
<td>295 ± 11</td>
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Fig. 3. Inclusions that are strongly immunoreactive with SOD1 antibodies are common features in transgenic mice expressing SOD1G93A, SOD1G85R, and SOD1G37R and resemble those seen in human patients with familial ALS. (A) Hematoxylin and eosin (H&E) staining of the ventral portion of a spinal cord from an end-stage mouse expressing mutant SOD1G85R. (B) The identical section as in (A), reacted with an antibody recognizing both mouse and human mutant SOD1. (C and D) A similar pair of sections to those in (A) and (B), except that they are from a mouse (obtained from Jackson Laboratories) developing motor neuron disease from expressing mutant SOD1G93A (2). (E) An inclusion in the spinal cord, detected with H&E, from a patient who died from disease mediated by a frameshift mutation at SOD1 position 126. (F) The identical section as in (E), reacted with an antipeptide antibody that recognizes human wild-type SOD1 but not the truncated mutant. Arrowheads in (B), (D), and (F) point to inclusions that are visible in (A), (C), and (E) and are intensely immunoreactive to SOD1 antibodies. Arrows in (B), (D), and (F) point to SOD1 immunoreactive aggregates that appear to contain less dense accumulations of SOD1, which may be the precursors of the more intensely stained aggregates. Scale bar, 10 μm.
motor and sensory axons degenerating at the end stage were comparable, as were the numbers of aggregates in motor neurons and astrocytes (Fig. 4A), each of which was immunoreactive for SOD1 (Fig. 4B, arrowheads), as well as more diffuse SOD1-containing aggregates (Fig. 4B, arrows).

Thus, changing the levels of wild-type SOD1 polypeptides and activity between none and six times the normal level of SOD1 did not affect the pathology, onset, or progression of disease mediated by familial ALS mutant SOD1G85R. These findings are incompatible with several proposed toxic properties of the mutants and bear directly on possible therapeutic strategies.

First, contrary to earlier in vitro evidence (6, 7, 26, 27), these results showed that motor neuron degeneration almost certainly did not arise from more efficient use of either peroxynitrite or H₂O₂. Peroxynitrite production [proposed to form through the spontaneous reaction of nitric oxide with superoxide (6)] should be substantially elevated by the absence of wild-type SOD1 and probably lowered by a more rapid removal of superoxide in mice with a sixfold elevation of wild-type SOD1, thereby accelerating or slowing disease, respectively. This was clearly not the case for mutant SOD1G85R. When coupled with the absence of biochemically detectable protein nitration in SOD1G37R (28) and SOD1G93A (29) mice, it seems unlikely that such enzyme-mediated nitration is a key toxic property, despite apparent increases in nitration that have been reported using immunocytochemistry in some cases of familial and sporadic ALS (19, 30) and in SOD1G93A mice (29). Similarly, although we cannot directly measure the levels of intracellular H₂O₂, one might predict an elevation or lowering of H₂O₂ caused by increasing or decreasing levels of wild-type SOD1. This in turn would accelerate or slow disease were the mutant to use H₂O₂ more efficiently than the wild type. On the other hand, it may be that the H₂O₂ produced in the active site from superoxide is the preferred substrate for aberrant catalysis. A sixfold molar excess of wild-type SOD1 would obviously reduce the access of the mutant to superoxide (through competition or by accelerated catalytic destruction of it, or both), whereas elimination of wild-type SOD1 would strongly enhance it. Both would predict changes in the rate of damage. But our evidence, along with the absence of evidence for predicted damaged products in SOD1G37R (28) and SOD1G93A (29) mice and in vitro evidence questioning whether the mutants do use H₂O₂ as a substrate (31), showed that this too was not the case.

Second, the fact that disease was not accelerated in the complete absence of wild-type SOD1 argues directly against the hypothesis (9, 32) that toxicity arises even in part from a reduction in normal SOD1 activity. Third, although we show that wild-type SOD1 was a component of aggregates in human disease, the wild-type protein was not required for aggregation, either for stabilizing the mutant or for contributing to aggregates nucleated by mutant SOD1.

Finally, the failure of increased SOD1 activity to ameliorate disease argues strongly against potential therapeutic attempts to slow disease by increasing superoxide scavenging activities using SOD1 mimetics. Indeed, the absence of influence of SOD1 activity on mutant toxicity challenges the idea that toxicity derives from (or is even linked to) oxidative stress arising from superoxide. Rather, mutant SOD1-containing aggregates that coincide with disease onset and that increase in abundance as disease progresses were found to be common to disease caused by different mutants. However, this cannot be the whole story concerning toxicity, because mutant-mediated defects (including a selective slowing of some cargoes of slow axonal transport) are seen at least 5 months before the appearance of aggregates (33); and a mating experiment strategy similar to that used here has proven that in the absence of neurofilaments, SOD1G85R mediates disease with a substantially delayed onset (34) and a markedly reduced frequency of SOD1-containing aggregates (33). Nevertheless, the present efforts support the idea that one aspect of toxicity may arise through an as-yet-unidentified chemistry mediated by the misfolded aggregated mutants or through loss by coprecipitation of an essential component or components.

References and Notes
5. L. I. Bruijn et al., ibid., 18, 327 (1997).
13. SOD1−/− mice (line 148) (5) were bred to mice overexpressing human wild-type SOD1 (SOD1 line 30) to six times the level of endogenous SOD1. SOD1−/− mice were bred with mice heterozygous for SOD1 deletion (SOD1−/+) to generate SOD1G85R, SOD1−/− mice, which were then bred with SOD1−/− mice to generate, in the same litters, SOD1G85R mice with zero, one, or two copies of the endogenous SOD1 gene, as well as normal nontransgenic mice.
15. Immunoblots (70 μg of total spinal cord homogenates) were incubated with polyclonal antipeptide antibodies generated against a sequence present in human but not mouse SOD1 or against antipeptide antibodies specific for mouse SOD1 (5). Signals were detected with 125I-labeled protein A and quantified by phosphorimaging.
23. S. Kato et al., ibid., p. 1089.
30. M. F. Beal et al., ibid., p. 646.
Regulation of Meiotic S Phase by Ime2 and a Clb5,6-Associated Kinase in Saccharomyces cerevisiae

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Cyclin-dependent kinase (Cdk) mutations that prevent entry into the meiotic cell cycle of budding yeast fail to block meiotic DNA replication, suggesting there may be fundamental differences between these pathways. However, S phase in meiosis was found to depend on the same B-type cyclins (Clb5 and Clb6) as it does in mitosis. Meiosis differs instead in the mechanism that controls removal of the Cdk inhibitor Sic1. Destruction of Sic1 and activation of a Clb5-dependent kinase in meiotic cells required the action of the meiosis-specific protein kinase Ime2, thereby coupling early meiotic gene expression to control of DNA replication for meiosis.

In eukaryotes, both mitotic chromosomal DNA replication (S phase) and chromosome segregation (M phase) are triggered by cyclin-dependent kinases (Cdks). In budding yeast, specific B-type cyclins activate the principal Cdk, Cdc28, to control entry into S phase. Late in G1, a series of events leads to activation of the S-phase promoting factor (SPF), a complex of Cdc28 with either of two similar B-type cyclins, Clb5 and Clb6 (1–3). Before S phase, the Clb5–6/Cdc28 kinase is maintained in an inactive state by its association with the CDK inhibitor (CDI) Sic1. Targeting of Sic1 for degradation requires activity of the Cdc28 kinase in conjunction with the G1-specific cyclins Cln1 and Cln2 (4–7). In mutants lacking Clb5 and Clb6, S phase occurs but is delayed, because other B-type cyclins (CLB1–4) that arise later in the cell cycle can also fulfill this function (8).

In meiosis, the G1- to S-phase transition is controlled differently. The temperature-sensitive CDK mutation that blocks mitotic S phase, cdc28-4, has no effect on meiotic S phase (9). Thus, Cdc28 might render mitotic DNA synthesis dependent on growth conditions, while being dispensable for meiotic S phase (10). One possibility is that the meiosis-specific kinase encoded by IME2, which has sequence similarity to Cdc28 and is required for meiotic S phase (11–13), replaces Cdc28 in an SPF-like function that is required in meiosis. To find out, we have reinvestigated genetic interactions that influence DNA replication in meiosis.

Yeast strains mutant for the G1-specific cyclins CLN1, -2, -3 are blocked in mitosis at G1 (14, 15) but form viable spores to the same extent as do wild-type (WT) cells (16, 17). Likewise, mutations eliminating components of transcription factors for the CLN1 and CLN2 genes (SBF and MBF) cause mitotic G1 arrest (18) but fail to block meiosis (16, 17). These findings are consistent with a differential control of S phase between mitosis and meiosis, but the results of introducing mutations in CLB5 and CLB6 (19) are not. Although a strain deleted for CLB6 is fully proficient in sporulation, a clb5Δ homozygote fails to sporulate (16) (Fig. 1C). Because different genetic backgrounds vary in sporulation efficiency, we also tested clb5Δ in two other strain backgrounds (W303 and YK) and obtained similar results (16, 19). Meiotic S phase (20) in a clb5Δ homozygote was delayed about 3 hours relative to the WT (Fig. 1B) and meiotic progression was blocked either in G2 (60% of cells) or in meiosis I (40% of cells) (Fig. 1, A and C). The delay of S phase did not result from delayed initiation of meiosis, for the increase in transcription of both IME2 (an early meiotic gene) and CLB6 occurred on schedule and to even higher levels than that in WT cells (Fig. 1D) (20). Assessment of intragenic recombination within the HIS4 locus revealed no His– progeny (21), indicating either that CLB5 is required for recombination or that recombinants are inviable. We conclude that CLB5 function is required for scheduled entry into S phase and that clb5Δ homozygotes, despite their capacity for extensive DNA replication, are unable to progress normally through subsequent stages of meiosis. These latter anomalies might reflect either qualitative defects in the newly replicated DNA or an additional requirement for CLB5 function in the transition from G1 to meiosis I. The finding that CLB5 is also activated by the G2-specific meiotic transcription factor Ndt80 (22) favors the latter explanation.

Because CLB6 is functionally redundant with CLB5 in mitosis (1–3), we also tested strains deleted for both genes (16). In both genetic backgrounds tested, sporulation failed to occur and DNA replication was impaired to a greater extent than in cells lacking CLB5 alone (Fig. 1, B and C), which indicates that either CLB5 or CLB6 can act to promote meiotic DNA synthesis. Flow cytometric profiles showed late and incomplete DNA replication, and further progression through the meiotic pathway did not occur (Fig. 1, A and B) (21).

Because both the Clb5 and Clb6 cyclins and the Ime2 kinase are required for meiotic DNA replication (Fig. 1) (11–13), they might constitute a single complex that promotes the G1–S transition in meiosis (Fig. 4B2). Alternatively, Ime2 might mediate entry into S phase indirectly by controlling removal of an inhibitor, such as Sic1 (Fig. 1B3). The target of this inhibitor would be an unspecified meiotic Cdk (designated X) that is activated by association with Clb5 and Clb6, and the role of Ime2 in meiosis would parallel that of Cln1, -2/Cdc28 in mitosis. In this case, deletion of SIC1 might render IME2 dispensable for DNA replication. Indeed, deletion of SIC1 in ime2Δ cells restored DNA replication to the level and kinetics observed for the control sic1Δ strain (Fig. 2). Thus, Sic1 acts by blocking entry into meiotic S phase, and Ime2, instead of promoting entry directly, may con-