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Timothy M. Miller, MD, PhD
Background: Therapies designed to decrease the level of SOD1 are currently in a clinical trial for patients with superoxide dismutase (SOD1)–linked familial amyotrophic lateral sclerosis (ALS).

Objective: To determine whether the SOD1 protein in cerebral spinal fluid (CSF) may be a pharmacodynamic marker for antisense oligonucleotide therapy and a disease marker for ALS.

Design: Antisense oligonucleotides targeting human SOD1 were administered to rats expressing SOD1<sup>G93A</sup>. The human SOD1 protein levels were measured in the rats' brain and CSF samples. In human CSF samples, the following proteins were measured: SOD1, tau, phosphorylated tau, VILIP-1, and YKL-40.

Participants: Ninety-three participants with ALS, 88 healthy controls, and 89 controls with a neurological disease (55 with dementia of the Alzheimer type, 19 with multiple sclerosis, and 15 with peripheral neuropathy).

Results: Antisense oligonucleotide-treated SOD1<sup>G93A</sup> rats had decreased human SOD1 messenger RNA levels (mean [SD] decrease of 69% [4%]) and decreased protein levels (mean [SD] decrease of 48% [14%]) in the brain. The rats' CSF samples showed a similar decrease in hSOD1 levels (mean [SD] decrease of 42% [14%]). In human CSF samples, the SOD1 levels varied a mean (SD) 7.1% (5.7%) after additional measurements, separated by months, were performed. The CSF SOD1 levels were higher in the participants with ALS (mean [SE] level, 172 [8] ng/mL; P < .05) and the controls with a neurological disease (mean [SE] level, 172 [6] ng/mL; P < .05) than in the healthy controls (mean [SE] level, 134 [4] ng/mL). Elevated CSF SOD1 levels did not correlate with disease characteristics in participants with ALS or controls with dementia of the Alzheimer type, but they did correlate with tau, phosphorylated tau, VILIP-1 and YKL-40 levels in controls with dementia of the Alzheimer type.

Conclusions: SOD1 in CSF may be an excellent pharmacodynamic marker for SOD1-lowering therapies because antisense oligonucleotide therapy lowers protein levels in the rat brain and rat CSF samples and because SOD1 levels in CSF samples from humans are stable over time.


MYOTROPIC LATERAL SCLEROSIS (ALS) is an adult-onset, neurodegenerative disease characterized by selective death of the upper and lower motor neurons of the brain and spinal cord. Symptoms include muscle atrophy, spasticity, paralysis, and eventual death from respiratory failure within 3 to 5 years of diagnosis. There are no adequate therapies. Although ALS mostly affects patients without a family history of the disease, 5% to 10% of ALS cases are familial ALS. Nearly 20% of familial ALS cases are caused by Cu/Zn superoxide dismutase (SOD1) gene mutations.<sup>1</sup> SOD1 is a ubiquitously expressed, cytosolic enzyme involved in the removal of superoxide. Although the mechanism is unclear, mutant SOD1 gains a toxic function independent of its normal enzymatic activity.<sup>2,3</sup>

The fact that mutant SOD1 causes disease by a toxic gain of function<sup>2,4</sup> suggests that lowering levels of mutant SOD1 could benefit patients with SOD1-linked ALS. Antibody-mediated lowering of SOD1,<sup>5</sup> small interfering RNA delivered to SOD1 by virus,<sup>6,8</sup> and antisense oligonucleotides delivered to SOD1<sup>9</sup> have thus far demonstrated that lowering SOD1 in transgenic SOD1 mouse and rat models delays SOD1-mediated disease.<sup>10</sup> Smith and colleagues<sup>8</sup> demonstrated the feasibility of the
bind messenger RNA (mRNA) in a sequence-specific manner. Antisense oligonucleotides are short DNA-like chemicals that may be part of sporadic ALS.14 Given these findings, we can attribute changes in CSF SOD1 levels to antisense oligonucleotide therapy rather than to the innate variability of SOD1 in the CSF.

An overlapping interest in SOD1 CSF levels in patients with ALS stems from the growing number of reports implicating SOD1 in the pathogenesis of sporadic ALS. Gruzman and colleagues12 found an SOD1-reactive protein (after chemical cross-linking) in participants with ALS not in controls. Antibodies that specifically recognize misfolded SOD1 revealed misfolded SOD1 in vulnerable spinal cord neurons of participants with ALS but not controls.13 Most interestingly, lowering SOD1 levels in astrocytes derived from sporadic participants with ALS reversed the toxicity of these same astrocytes when cocultured with motor neurons, again implying that SOD1 may be part of sporadic ALS.14 Given these findings, we also examined SOD1 protein levels in CSF samples as a potential biomarker for sporadic ALS.

QUANTIFYING ANTISENSE OLIGONUCLEOTIDE, mRNA, AND PROTEIN LEVELS

An enzyme-linked immunosorbent assay was conducted to confirm the dosage of antisense oligonucleotide 333611 in the rat brain. The total RNA was extracted (Qiagen 74106) from rat tissues, and human SOD1 and rat SOD1-mRNA expression were measured relative to rat cyclophilin by use of real-time quantitative polymerase chain reaction on the Applied Biosystems 7500 Fast Real-Time PCR System. Primers and probes were ordered from Integrated DNA Technologies. Primer and probe sequences for human SOD1 were as follows: forward, 5’-TGCAATCATTGCGCGCA-3’; reverse, 5’-TTCCTTCATTTC-CACCTTGGCC-3’; probe, 5’/-56-FAM/ACGTGTCATTCCAT-GAAAACGACATGGCTT36-TAMT ph-3’. Primer and probe sequences for rat SOD1 were as follows: forward, 5’-CGGATAGAAGAGGCGATTTG-3’; reverse, 5’TGGCCA-CACCGTCTCTTT-3’; probe, 5’/-56-FAM/AGACCTGGCAAT-GTGGCTGCTG36-TAMT ph-3’. Primer and probe sequences for rat cyclophilin were as follows: forward, 5’-CCACCGTGTCCTTGACA-3’; reverse, 5’-AACAGCCTG-GAGCAGAGGC-3’; probe 5’/-56-FAM/CACCGTCTTGAGCGAGC36-TAMT ph-3’. Right temporal-parietal brain sections from rats underwent dounce homogenization in lysis buffer. Total protein measurements were quantified by use of a bicinchoninic acid assay (Pierce 23227). Human SOD1 protein was quantified by use of a Cu/Zn SOD1 enzyme-linked immunosorbent assay kit (eBioscience BMS222MS). Hemoglobin was detected by use of an enzyme-linked immunosorbent assay (Bethyl Laboratory, Inc, E80-135).

HUMAN CSF SAMPLES AND PATIENT DATA SETS

The CSF samples obtained from 88 healthy controls, 89 controls with a neurological disease (55 with Alzheimer disease [AD], 19 with multiple sclerosis, and 15 with peripheral neuropathy) and 93 participants with ALS were donated by the Charles F. and Joanne Knight Alzheimer’s Disease Research Center of Washington University in St Louis, Missouri, and the Northeast ALS Consortium. The Northeast ALS Consortium and the Knight Alzheimer’s Disease Research Center, respectively, provided the ALS and AD biomarker and clinical data sets. Samples of CSF were obtained from 14 patients from University of Pittsburgh, Pennsylvania, at repeated time points ranging from 7 to 48 months after the initial lumbar puncture. These samples were obtained by the same physician at the same time of day (afternoon). Institutional review board–approved consent was obtained prior to the collection of CSF samples at Massachusetts General Hospital in Boston, Washington University, and the University of Pittsburgh. All CSF samples were immediately placed on ice, centrifuged at 500g at 4°C, and then immediately frozen at −80°C. All CSF samples were clear and colorless.

STATISTICS

Data were analyzed using a 2-tailed t test. P values of less than .05 were considered to be statistically significant. The strength of correlation was measured by the Pearson correlation coefficient.
We found that the SOD1 protein level in CSF was decreased a mean (SD) of 42% (14%) (Figure 1B), respectively, in the right frontal cortex. To test whether this decreased level of SOD1 protein in the brain would be reflected in the cerebrospinal fluid (CSF), we measured the SOD1 protein level in the CSF. The average total time participants were observed was 23 months. Although there was substantial variability among individuals’ absolute levels of CSF SOD1, there was surprisingly little variability within individuals over time (mean [SD] variation, 7.1% [5.7%]). The maximum variation between any 2 points for a given participant was 21%.

Figure 1. Results of antisense oligonucleotide therapy in rats. Antisense oligonucleotides complementary to SOD1 decreased human SOD1 levels in the brain and cerebral spinal fluid (CSF) of rats. SOD1G93A rats were dosed with saline (n = 12) or an antisense oligonucleotide complementary to human SOD1 (n = 7) for 1 month, and then the pumps were removed. Two weeks later, CSF and brain samples were harvested. SOD1 messenger RNA (mRNA) was analyzed by use of quantitative polymerase chain reaction (A). SOD1 protein levels in brain (B) and CSF (C) samples were analyzed by use of an enzyme-linked immunosorbent assay and correlated (D). Values are expressed as mean values. Error bars indicate SD. *P < .05, determined by use of the t test. Oligo indicates antisense oligonucleotide.

Figure 2. SOD1 levels in cerebral spinal fluid (CSF) samples obtained from humans. The CSF SOD1 level was determined for individual participants from lumbar punctures completed at the indicated times: 11 participants with amyotrophic lateral sclerosis (each closed symbol or asterisk represents an individual participant with amyotrophic lateral sclerosis), 2 healthy controls (open squares), and 1 control with a neurological disease (open triangle). The average total time participants were observed was 23 months. Although there was substantial variability among individuals’ absolute levels of CSF SOD1, there was surprisingly little variability within individuals over time (mean [SD] variation, 7.1% [5.7%]). The maximum variation between any 2 points for a given participant was 21%.

To use SOD1 as a pharmacodynamic marker, another important consideration is the variability of SOD1 in CSF in the same individual over time. In 14 participants who had CSF samples obtained at various time points over 7 to 48 months (average time between CSF samples, 8 months; average total time participants were observed, 23 months), we measured CSF SOD1 protein levels (Figure 2). Although there was substantial variability among individuals’ absolute levels of CSF SOD1, there was surprisingly little variability within individuals over time (mean [SD] variation, 7.1% [5.7%]). The maximum variation between any 2 points for a given participant was 21%. Among the 55 total time points, there were 4 instances of variability greater than 15%. Given that within the same individual, SOD1 varies little over time, CSF SOD1 measurements are likely to accurately reflect the effects of an SOD1-lowering therapy rather than the natural variability of SOD1 metabolism or of the measurement itself.

To better evaluate SOD1 as a potential biomarker for sporadic ALS, we measured CSF SOD1 and total protein levels in 93 participants with ALS, 88 healthy controls, and 89 controls with a neurological disease. The patient characteristics are shown in Table 1. Although all CSF samples appeared 100% clear of any blood contamination by visual inspection, we wanted to further exclude microscopic blood contamination of CSF because red blood cells are a well-known source of SOD1. To do so, we measured CSF hemoglobin levels. The maximum CSF hemoglobin level in any patient was 9000 ng/mL. We next measured the SOD1 level in human blood. We found 1 ng of SOD1 per 100 000 ng of hemoglobin.
Table 1. Characteristics of Participantsa

<table>
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</table>

Abbreviation: ALS, amyotrophic lateral sclerosis.

a Of these 89 controls, 55 had dementia of the Alzheimer type, 19 had multiple sclerosis, and 15 had peripheral neuropathy.

Figure 3. A and B, SOD1 protein levels in cerebral spinal fluid (CSF), which are elevated in participants with amyotrophic lateral sclerosis (ALS) and controls with a neurological disease. In the CSF samples from 88 healthy controls, 89 controls with a neurological disease, and 93 participants with ALS, the SOD1 levels were measured by use of an enzyme-linked immunosorbent assay (A), and total protein levels were measured by use of a bicinchoninic acid assay (B). Values are expressed as mean values. Error bars indicate SE.* P < .05. C, The ratio of SOD1 to total protein was also compared. Of the 89 controls with a neurological disease, 55 received a clinical diagnosis of dementia of the Alzheimer type.

Therefore, even in our most “contaminated” sample, the contribution to the SOD1 measurement from hemoglobin contamination was approximately 0.1%. Consistent with this, hemoglobin levels did not correlate with CSF SOD1 levels (R = -0.016 and P = .92 for healthy controls, R = -0.159 and P = .24 for controls with a neurological disease, and R = -0.059 and P = .72 for participants with ALS).

The CSF SOD1 levels were significantly elevated in controls with a neurological disease and participants with ALS (Figure 3). Total protein levels were significantly elevated in participants with ALS (Figure 3), as has been reported previously.17,18 The ratio of SOD1 protein to total protein in CSF was significantly elevated in controls with a neurological disease and participants with ALS (Figure 3C). Thus, although the elevation of SOD1 is particularly interesting for ALS because SOD1 mutations cause ALS, the similar increase in controls with a neurological disease suggests that the increase is either non-specific for ALS or specific for the particular set of diseases studied.

Correlations with participant characteristics have the potential to explain some of these changes. As would be expected from previous studies,19 CSF total protein levels correlated moderately (R = 0.3, P ≤ .05) with increased age in healthy controls (Table 2). Total protein levels did not correlate with age for controls with a neurological disease or participants with ALS. The CSF SOD1 levels did not correlate with age in either controls or participants with ALS, but they did correlate modestly with age for controls with a neurological disease (R = 0.41, P < .50) (Table 2). Previous work with 11 participants with ALS and 19 controls has suggested a difference between men and women in SOD1 CSF levels.20 In our CSF samples, the mean (SE) protein levels for men were 142 (47), 173 (67), and 167 (63) ng/mL for healthy controls, controls with a neurological disease, and participants with ALS, respectively. For women, the mean (SE) levels were 127 (39), 174 (85), and 189 (78) ng/mL for healthy controls, controls with a neurological disease, and participants with ALS, respectively. None of the differences between men and women were significant (P = .10, .95, and .16 for healthy controls, controls with a neurological disease, and participants with ALS, respectively).

For participants with ALS, we hypothesized that the CSF SOD1 levels would be elevated in those with more severe disease as determined by ALS Functional Rating Scale at the time the CSF sample was obtained or in participants with a more rapidly progressing ALS as determined by the ALS Functional Rating Scale change per month. As shown in Figure 4, neither of these hypotheses are correct. The total CSF protein level also did not correlate with the ALS Functional Rating Scale (data not shown).

Of the 89 controls with a neurological disease, 55 were participants with very mild (Clinical Dementia Rating [CDR] scale of 0.5) or mild (CDR scale of 1) dementia of the Alzheimer type. Similar to participants with ALS, these 55 participants had SOD1 CSF levels that did not correlate with disease severity (CDR scale of 0.5 or 1). Because biomarkers for AD have been extensively studied, we analyzed previously described markers of disease in participants with mild dementia (CDR scale of 0.5 and 1) and age-matched, neurologically normal controls (Table 3). The SOD1 CSF levels were either weakly correlated or not correlated with measures of amyloid plaque load, including Aβ42 CSF levels and the mean cortical binding potential of Pittsburgh Compound B, an imaging compound that specifically assesses the fibrillar amyloid plaque burden.21,22 Markers of neuronal damage (tau, phosphorylated tau, and VILIP-1)23,24 and in-
flammmation (YKL-40)25 were more strongly correlated with CSF SOD1 levels (Table 3), although more so in the controls and participants with a CDR scale of 0.5 than in participants with a CDR scale of 1, with the exception of YKL-40, which showed a weak correlation with a CDR scale of 0 and a stronger correlation with a CDR scale of 1. These data suggest that increased SOD1 CSF levels in patients with neurodegenerative disease are correlated with an increased degree of neuronal damage and inflammation.

**COMMENT**

To our knowledge, we demonstrated, for the first time, a strong correlation between knockdown of a target in the brain and knockdown of the same target protein in CSF. For designing therapeutic trials for SOD1 and other neurodegenerative disease proteins, demonstrating knockdown in the CSF is a key piece of information because now, based on these animal data, we have confidence that SOD1 CSF will be an appropriate fluid for determining a pharmacodynamic response. The ability to obtain CSF samples, which is a relatively routine procedure, will greatly enhance our ability to determine whether the antisense oligonucleotides indeed decrease SOD1 levels in the central nervous system.

Equally important for consideration of SOD1 as a pharmacodynamic marker is the finding that there is little variation in CSF SOD1 levels when measured repeatedly, with an average variation of 7% between time points. This finding is consistent with the lack of correlation between CSF SOD1 levels and ALS disease severity and progression, suggesting that once a patient becomes sick, his or her SOD1 level remains stable throughout the disease course. Based on our finding that the CSF SOD1 level decreases by a mean (SD) of 42% (14%) in antisense oligonucleotide-treated rats and based on a mean (SD) variation of 7.1% (5.7%) from point to point in humans, we estimate that we have a more than 95% chance of seeing a 40% or more reduction in CSF SOD1 levels in as few as 6 participants with mutant SOD1, familial ALS. We recognize that we may or may not obtain the same robust knockdown in participants in a phase II trial as we see in rodents, and therefore we will need a larger sample size (eg, 20-30 participants). Because we successfully recruited participants for our phase I trial from this same population, we consider 20 to 30 participants an achievable goal for such a phase II trial.

One remaining issue for planning a pharmacodynamic study of modulating CSF SOD1 levels is the half-life of the protein. Using hydrogen-deuterium exchange and mass spectrometry, Farr et al26 suggested that the tissue half-life of a SOD1-YFP fusion protein in a mouse is approximately 22 days. Whether the SOD1 half-life in CSF is the same as in tissue is an important, ongoing question that may be addressed by using stable isotope-linked kinetics, a method developed to test a protein’s synthesis and half-life in CSF in research subjects.27 In addition, it remains unclear whether the process of YFP fusion could affect a protein’s half-life.

Given the recent data suggesting that SOD1 may contribute to sporadic ALS, we explored the possibility of whether the SOD1 level is specifically increased in sporadic ALS but not in other diseases. Our data do not support this hypothesis. We found increased levels of SOD1 in the CSF of controls with a neurological disease and participants with ALS; thus, an increased SOD1 level is not a specific biomarker for ALS. We also considered whether CSF SOD1 levels might vary according to ALS disease progression or disease severity, but we found no correlation. In participants with dementia of the Alzheimer type, for which biomarkers of disease have been extensively evaluated, we also compared CSF SOD1 levels with Aβ, tau, phosphorylated tau, VILIP-1, and YKL-40...
levels. Like the SOD1 CSF levels in participants with ALS, the SOD1 CSF levels in participants with dementia of the Alzheimer type did not correlate well with severity of disease. In addition, SOD1 levels did not correlate strongly with Aβ levels, suggesting that SOD1 is not a component of the abnormal Aβ cascade of AD. We did, however, find correlations with tau, phosphorylated tau, and VILIP-1, which are markers of neuronal damage. We also found a strong correlation with YKL-40, likely a marker of inflammation. We conclude that increased SOD1 levels in the CSF of participants with AD and participants with ALS are likely associated with increased neuronal damage and inflammation. Because our controls with a neurological disease were dominated by AD, it remains possible, though we believe unlikely, that an increased SOD1 level in the CSF is specific to AD and ALS and is not found in other neurodegenerative diseases.

In our study, we found increased total protein levels in the CSF of participants with ALS. This is similar to previous reports from Guiloff et al17 (38 cases) and Norris28 (385 cases). On the other hand, Younger et al,18 in a series of 120 patients, did not find increased total CSF protein levels. The reason for increased CSF protein levels in some series remains unclear. Neither our study nor other reported studies found correlations of CSF protein levels with other disease measures.

For ALS, specific SOD1 posttranslational modifications or an aggregation state may be a more important biomarker than total SOD1 levels. Although total SOD1 levels are not likely to be a biomarker for sporadic ALS, we remain enthusiastic about ongoing studies to examine whether different properties of SOD1 (eg, misfolded SOD1 in the CSF) may be a biomarker for a subset of sporadic ALS, as suggested by studies of postmortem material from participants with ALS.12-14 However, a recent article by Zetterström and colleagues,29 who used antibodies that recognize misfolded SOD1, showed no difference in misfolded SOD1 in the CSF samples obtained from participants with ALS vs controls, even when comparing participants with ALS who had known SOD1 mutations.

The data presented herein demonstrate that the CSF SOD1 level is stable and that knockdown of SOD1 in the brain results in reduced SOD1 levels in the CSF. We propose that SOD1 in the CSF is likely to be a useful pharmacodynamic marker for therapies designed to lower SOD1 levels in the brain and spinal cord.
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Online-Only Material: The eFigure is available at http://www.jamaneo.com.

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REFERENCES