Mutant superoxide dismutase-1 indistinguishable from wild-type causes ALS

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A reason for screening amyotrophic lateral sclerosis (ALS) patients for mutations in the superoxide dismutase-1 (SOD1) gene is the opportunity to find novel mutations with properties that can give information on pathogenesis. A novel c.352C>G (L117V) SOD1 mutation was found in two Syrian ALS families living in Europe. The disease showed unusually low penetrance and slow progression. In erythrocytes, the total SOD1 activity, as well as specific activity of the mutant protein, was equal in carriers of the mutation and family controls lacking SOD1 mutations. The structural stabilities of the L117V mutant and wild-type SOD1 under denaturing conditions were likewise equal, but considerably lower than that of murine SOD1. As analyzed with an ELISA specific for misfolded SOD1 species, no differences were found in the content of misfolded SOD1 protein between extracts of fibroblasts from wild-type controls and from an L117V patient. In contrast, elevated levels of misfolded SOD1 protein were found in fibroblasts from ALS patients carrying seven other mutations in the SOD1 gene. We conclude that mutations in SOD1 that result in a fully stable protein are associated with low disease penetrance for ALS and may be found in cases of apparently sporadic ALS. Wild-type human SOD1 is moderately stable, and was found here to be within the stability range of ALS-causing SOD1 variants, lending support to the hypothesis that wild-type SOD1 could be more generally involved in ALS pathogenesis.

INTRODUCTION

The amyotrophic lateral sclerosis (ALS) syndrome is characterized by adult-onset progressive loss of upper and lower motor neurons, resulting in paralysis and inevitable death from respiratory failure. Although most of the cases appear to be sporadic amyotrophic lateral sclerosis (SALS), ~10% of patients report a familial predisposition (denoted FALS). The 15 identified ALS-associated genes explain half of the familial cases, and mutations in them have occasionally also been reported in apparently SALS cases (1). Mutations in the gene encoding superoxide dismutase-1 (SOD1) are found in ~6% of all ALS cases (2,3). Some 167 mutations have been found (http://alsod.iop.kcl.ac.uk/) and

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they confer an enigmatic cytotoxic gain of function on the enzyme (4,5). The neurotoxicity is assumed to stem from reduced structural stability of the SOD1 protein. Most of the over 100 mutant SOD1s that have been tested show instability in vitro (6–8). In the few cases that have been studied, the levels of SOD1 in the CNS have mostly been low, suggesting increased recognition and degradation by the quality control systems (9).

Erythrocytes are easily accessible and therefore more commonly analyzed. They also degrade misfolded proteins, lack protein synthesis and are on average 60 days old. In a study of erythrocytes from heterozygous carriers of SOD1 mutations, the content of mutant SOD1 protein was found to vary from undetectable to 58% of that of wild-type SOD1 (10). The most structurally stable of the SOD1 mutant proteins found so far found is the D90A (11). This is also the only SOD1 mutation to be associated with recessive inheritance (5). The SOD activity in erythrocytes of D90A-homozygous patients is 91% of controls (5,12). The similarity to the wild-type SOD1 suggested low toxicity, and has been proposed to be the reason for the recessive heredity, the uniform phenotype and the slow progression (5,13).

There is mounting evidence that wild-type human SOD1 may be neurotoxic and participate in ALS pathogenesis. Inclusions containing aggregated SOD1 are considered to be hallmarks of mutant SOD1-provoked ALS (14), but can with a set of antibodies specific for misfolded human SOD1 also regularly be demonstrated in motor neuron somas (15) and glial cell nuclei (16) of ALS patients lacking SOD1 mutations. Other antibodies reactive with an epitope in mutant SOD1 or in misfolded SOD1 have been found to stain motor neuron somas (17) and axons (18) in some patients with sporadic ALS. In addition, mice that overexpress wild-type human SOD1 have a normal life span but show a major late loss of spinal motor neurons (19,20). Such overexpression also exacerbates disease in mice expressing mutant SOD1s (19,21,22). Finally, wild-type human SOD1 by itself has a moderate structural stability. Under denaturing conditions, it is only marginally more stable than the D90A mutant (12), and less stable than murine wild-type SOD1 (23). These earlier findings suggest that the stability of wild-type SOD1 could be in the same range as those of variants that can cause ALS. We here report a novel SOD1 mutant that is as stable as the human wild-type protein.

RESULTS

Genotyping and genealogy

As part of an epidemiological study of ALS genetics in Sweden, blood samples were collected from ALS patients and analyzed for SOD1 mutations by sequencing SOD1 and analyzing the SOD activity in erythrocytes as described previously (5). A novel non-synonymous missense variant c.352C>G was identified resulting in a leucine for valine substitution in codon 117 (L117V) (NM_000454.4: c.352C>G_1332757776). The index patient had an age of onset of 35 years and was initially diagnosed as having SALS. Both of her parents and all six siblings (aged between 29 and 46 years) are without symptoms. However, the finding of an SOD1 mutation prompted further investigation of the family and revealed that a maternal uncle (living in Germany) and the uncle’s cousin (living in Switzerland) also suffered from ALS, and that at least three other members had died from ALS in Germany or Syria. The family originates from a remote area of northeastern Syria bordering on Turkey, and came to Europe because of religious persecution. A pedigree and a summary of clinical data are given in Supplementary Material, Fig. S1 and Table S1.

Blood samples were collected from 14 available members of the family across Europe and analysis revealed that the ALS patient III-8 is homozygous for the L117V mutation while ALS-patients III-12 and IV-3 are heterozygous for L117V. Four unaffected members of the family were also found to be heterozygous for L117V (Supplementary Material, Table S2). Another family (pedigree #2) from the same area of Syria but now living in Germany was then identified. In this family, one member developed disease at age 39 and had a diagnosis of ALS. DNA analysis showed the patient to be heterozygous for L117V. Fifty-two control individuals from the same geographic area were screened for L117V. None carried the mutation. Moreover, collectively we have since 1993 genotyped over 7000 ALS patients and controls from around the world and have not found the L117V mutation before, which suggest that it is not simply a common polymorphism. To exclude the possibility that the patients were carriers of a mutation in another gene associated with ALS, at least one of III-8, III-12 and IV-3 from pedigree #1 were also tested for other ALS-associated genes. This screening was negative with the exception of III-12 who was also found to carry a novel PGRN missense mutation (c.1228T>A; G410S). This mutation was not found in the other patients and did not therefore segregate with disease. The symptoms and progression of III-12 do not deviate from the other L117V patients and the significance of the PGRN mutation for the disease is thus questionable.

L117V mutant SOD1 is indistinguishable from the wild-type enzyme

A remarkable finding was that the heterozygous carriers of the L117V mutation showed normal SOD activity in erythrocytes (Table 1 and Supplementary Material, Table S2). The activity in the homozygous ALS patient was slightly lower (42 U/mg hemoglobin) but well within the range observed in controls and ALS patients lacking SOD1 mutations (5). To exclude the possibility that the normal SOD activity could be explained by increased specific activity of the L117V-mutant protein, SOD1 protein was also measured by ELISA in the

Table 1. SOD1 activities in erythrocytes

<table>
<thead>
<tr>
<th>Genotype SOD1</th>
<th>U/mg hemoglobin</th>
<th>ng SOD1 protein/U</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heterozygous L117V</td>
<td>61.3 ± 7.6 (n = 8)</td>
<td>5.1 ± 0.7 (n = 8)</td>
</tr>
<tr>
<td>Homozygous wild-type</td>
<td>61.6 ± 3.1 (n = 7)</td>
<td>5.2 ± 0.5 (n = 5)</td>
</tr>
</tbody>
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Total SOD1 activities and specific activities of SOD1 in erythrocytes from family individuals wild-type or heterozygous for L117V in the SOD1. The values presented are means ± SD.

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hemolysates. The calculated specific activities (ng SOD1 protein per unit of SOD activity) were, however, also found to be the same in the heterozygous L117V carriers and wild-type family controls (Table 1).

The properties of the L117V mutant were further examined by western immunoblot of hemolysates and fibroblast extracts, and compared with samples from patients with other SOD1 mutations (Fig. 1A and B). Although some other SOD1 mutants showed altered electrophoretic migration, the results for the L117V mutant were identical to the wild-type controls, and no aberrant bands were seen.

These findings suggested a high structural stability of the L117V mutant. This was further examined under strongly denaturing conditions (Fig. 2). As previously found (12,23), the murine SOD1 is far more stable than wild-type human SOD1, which in turn showed a moderate stability equal to that of the L117V mutant.

To explore the in vivo properties of the L117V mutant, the amounts of misfolded SOD1 were measured with a misELISA in fresh extracts of fibroblasts from controls and carriers of SOD1 mutations (Table 2). The L117V and D90A extracts had the same levels of misfolded SOD1 as the controls, whereas increased levels of misfolded SOD1 were found in extracts of fibroblasts from carriers of seven other SOD1 mutations.

**DISCUSSION**

Three of the patients in the families originally had a diagnosis of sporadic ALS. The finding of the SOD1 mutation prompted further genealogical investigation, revealing a familial predisposition for ALS across four countries. The L117V SOD1 mutation co-segregated with the disease, was not found in any controls from the same geographic area, and is most likely causing the disease in the families. Some older unaffected family members were also found to carry the L117V mutation, suggesting that it is associated with low disease penetrance. Interestingly, the transmitting unaffected individual in most cases is a woman, in keeping with the fact that ALS is more common in men than women. The special circumstances (refugees and an ethnic minority) and the fact that they settled in three different European countries further complicated the revelation of familial disease. Twenty-three of the earlier reported 166 SOD1 mutations have been associated with reduced disease penetrance, and this may mask the hereditary predisposition (3,24–26). Another notable finding is the uniform phenotype, with the onset of first symptoms in the lower extremities and a relatively slow disease progression in all patients, including patient III-8 who is homozygous for L117V. Previous reports on single patients homozygous for the SOD1 mutations L84F (27), N86S (28 and A.N. Basak,
unpublished result, 2010) or L126S (29) in highly inbred families (where also heterozygous carriers have developed ALS) have shown a more aggressive ALS phenotype in homozygous compared with heterozygous mutant carriers, suggesting a dose–response effect. However, the L84F, N86S and L126S all result in unstable mutant SOD1 proteins (11), and there is some correlation between the degree of instability and short survival time (10,11,30). Erythrocytes actively degrade misfolded proteins, lack protein synthesis and are on average 60 days old (31,32). As a result, unstable SOD1 mutants become undetectable and only mutants with stabilities close to that of the wild-type enzyme will show high SOD activities in erythrocytes. Over 90% of the enzymic activity of D90A mutant SOD1 remains in erythrocytes (5). There is very low disease penetrance in D90A-heterozygous carriers, and ALS in D90A-homozygous individuals shows a uniform phenotype and a slow progression (13). In concurrence, the fully stable L117V mutant SOD1 causes low disease penetrance, slow progression and a uniform phenotype with lower-limb onset—characteristics that may be typical of stable SOD1 mutants. That homo- and heterozygous patients with a SOD1 mutation co-exist in the same family has never been reported before. The disease was not obviously more aggressive in the homozygous individual. Speculatively, the neurotoxicity exerted by the wild-type-like L117V mutant SOD1 in the homozygous patient III-8 could be close to the combined toxicity of L117V mutant and wild-type SOD1 in the heterozygous patients. An extra copy of the fully stable L117V mutant protein perhaps makes little difference. In this context, it would be of interest if patients with Trisomy 21 Down’s syndrome with triplication of the SOD1 gene have increased risk of ALS. Unfortunately, we have found no epidemiological study on the prevalence of ALS in patients with Down’s syndrome.

Our studies of the biological samples failed to reveal any differences between the L117V mutant and wild-type human SOD1 (Figs 1 and 2 and Tables 1 and 2). The L117 position is located early in β-strand 7 of the core β-barrel of the SOD1 subunit, and is not strongly conserved (33). Of 57 reported eukaryotic SOD1 sequences, the amino acids found at position 117 are 18 leucines, 20 methionines, 12 valines, 2 phenylalanines and 4 isoleucines (www.ebi.ac.uk). Thus, the substitution to the smaller valine could be expected to have limited effect on the structure of the SOD1 subunit, although subtle effects not captured in the present analyses might present. One such property with relevance for ALS could be the propensity to form aggregates (34).

In other neurodegenerative conditions such as Alzheimer’s, Parkinson’s and Creutzfeld-Jacob’s diseases, some of the proteins found mutated in patients with a family predisposition are also thought to be involved in the pathogenesis in patients lacking such mutations (35). Could the same situation pertain to ALS? Neuronal cytosolic inclusions containing TAR DNA-binding protein 43 (TDP43) are found in patients carrying mutations in the TARDBP gene, but also in patients carrying mutations in other ALS-causing genes as well as in apparently sporadic ALS patients without mutations in any of these genes including TARDBP (36). Based on such findings, it has been suggested that TDP43 more generally might be involved in ALS pathogenesis. The disease caused by mutations in SOD1 has been regarded as an entity separate from ALS in general, since TDP43 inclusions are not found in the motor neurons (36). An antibody reactive with misfolded human SOD1 was recently found to label motor axons in a carrier of a fused in sarcoma (FUS) mutation as well as in sporadic ALS patients displaying cytosolic TDP43-immunoreactive inclusions (18). Moreover, cytosolic overexpression of human mutant TDP43 or FUS and wild-type TDP43 in cultured human neuroblastoma cells was found to induce misfolding of the endogenous wild-type human SOD1. Thus, misfolding of wild-type human SOD1 might occur secondarily to TDP43 or FUS pathology. Since most human SOD1 mutants owing to reduced structural stabilities are prone to misfold spontaneously, the findings might explain the absence of TDP43-immunoreactive inclusions in so far examined ALS patients carrying SOD1 mutations. Hypothetically, toxicity caused by misfolded human SOD1s might be a final common pathway in ALS pathogenesis.

The discovery of the L117V mutation adds significantly to our understanding of the role of SOD1 and its mutations in ALS. Relative to the murine SOD1, the human protein shows moderate structural stability. The human L117V mutation has here been found to be equally stable, and D90A SOD1 is only marginally less stable (23). The L117V mutation shows reduced ALS penetrance and the D90A mutation is mostly associated with recessive heredity. These findings suggest that the stability of the wild-type human SOD1 is indeed in the range of stability of variants that can cause ALS. Overall, there is a large variation in penetrance and disease phenotypes within and between families carrying SOD1 mutations (24–29), suggesting that there are genetic, environmental and/or lifestyle factors that influence the susceptibility to SOD1-induced neurotoxicity. Possibly such factors occasionally induce the wild-type SOD1 to cause ALS.

### PATIENTS AND METHODS

#### Human materials

We studied two Syrian families that had emigrated to Sweden, Germany and Switzerland in the 1980s because of persecution.
in Syria. Both families originated in the same region, close to the Syrian-Turkish border, and earlier generations of the family had lived on the Turkish side of the border. EDTA-anticoagulated blood was collected from the ALS patients and adult family members. Fifty-two control samples were obtained from the same ethnic group living on the Turkey side of the border or living in western Europe. A skin biopsy was obtained from a Swedish L117V ALS patient and 10 ALS patients (9 with other SOD1 mutations, 1 without) and two controls (one healthy and one with spinobulbar muscular atrophy). All specimens were collected after obtaining written informed consent and in accordance with the Declaration of Helsinki (WMA, 1964). The project was approved by medical ethical review boards in Germany, Italy, Turkey and Sweden.

Genotyping
Genomic DNA was extracted from buffy coat cells with the NUCLEON BACC2 DNA extraction kit (GE Healthcare, Piscataway, NJ, USA) or the FlexiGene DNA kit (QIAGEN, GmbH, Germany) according to the manufacturers’ protocols. All five exons and at least 30 bp of flanking intron sequences were amplified with the AmpliTaq Gold kit (Applied Biosystems, Foster City, CA, USA), sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and read in an Applied Biosystems 3730 DNA Analyzer. The reactions were analyzed with SeqScape v2.5 or in Sequencher v1.0 (Applied Biosystems) (5). At least one patient in Family #1 was also genotyped for mutations in the androgen-receptor gene, ANG, ALS2, SETX, FIG4, VCP, CHMP2B, FUS/TLS, OPTN, PGRN, SIGMAR1, TARDBP, UBQLN2, VAPB and C9ORF72 genes (details available upon request). The genetic analyses were performed in Umeå, Tübingen and Milan. Some analyses were performed in duplicate or triplicate.

Human skin fibroblast cell lines
The fibroblast lines were established from skin biopsy specimens using standard procedures. The cells were grown to confluence, then passaged and stored frozen. For SOD1 protein analysis, the cells were grown to near confluence, washed in isotonic NaCl solution, briefly exposed to trypsin, and then harvested in APBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4 and 1.4 mM KH2PO4) containing complete protease inhibitor cocktail with EDTA (Roche Diagnostics, Indianapolis, IN, USA) and 40 mM iodoacetamide to alkylate cysteine residues. They were then homogenized by sonication using a Sonifier Cell Disruptor (Branson, Danbury, CT, USA), and centrifugated at 20 000 g, and the supernatants were collected for analysis.

SOD1 activity and western immunoblot analysis
The SOD1 activity was analyzed with the direct assay using KO2 (37). In brief, the first-order dismutation of \( \text{O}_2^- \) derived from KO2 catalyzed by SOD is followed at 250 nm in a spectrophotometer. One unit is defined as the activity that brings about a dismutation at a rate of 0.1 s\(^{-1}\) in 3 ml of buffer at pH 9.5. The amounts of total protein in the fibroblast homogenates were determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, USA) and the amount of hemoglobin in the hemolysates were determined with a standard cyanomethemoglobin assay (br Bioreagen AB, Ellös, Sweden). The western immunoblots were performed as described previously (38, 39). The volumes loaded were adjusted so that the amount of protein or hemoglobin was equal in all samples. The primary (polyclonal rabbit) antibody used was raised to a peptide corresponding to amino acids 57–72 in the human SOD1 sequence. The chemiluminescence of the blots was recorded in a ChemiDoc apparatus and the amount of SOD1 protein was determined using Quantity One software (Bio-Rad).

Analysis of misfolded and total SOD1 by ELISA
The sandwich ELISA specific for misfolded SOD1 (misELISA) used the rabbit antibody raised against amino acids 57–72 of human SOD1 as the primary antibody and a goat anti-apo human SOD1 as the secondary antibody (35). The anti-peptide antibody lacks reactivity with native SOD1 but reacts avidly with misfolded SOD1 species (15, 16) and the anti-apo-SOD1 antibody has a reactivity that is 8-fold higher for misfolded SOD1 than for native SOD1. The analyses were carried out with fresh fibroblast extracts, since freezing and thawing leads to (measurable) further denaturation of SOD1. For analysis of total SOD1 by ELISA, rabbit and goat antibodies raised to native SOD1 were used as primary and secondary antibodies, respectively, as previously described (39).

Stabilities of human and murine SOD1 proteins
Pools of packed EDTA-anticoagulated erythrocytes from three C57Bl/six mice, three wild-type family controls and the homozygous L117V ALS patient were mixed with 1.6 volumes of a 37.5/62.5 (vol/vol) mixture of chloroform/ethanol at –20°C to precipitate and remove hemoglobin. After vortexing and centrifugation (2500 g, 10 min), 200 μl of the upper SOD1-containing phase was mixed with 400 μl 3.75 M guanidinium chloride in 0.1 M Na HEPES, pH 7.4, with 3 mM DTPA and incubated at 37°C. The denaturation was stopped after different times by addition of 50-μl aliquots of the mixture to 400 μl 50 mM Na HEPES, pH 7.4, with 0.25% BSA followed by analysis of SOD activity in triplicate.

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG online.

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