Disulphide-reduced superoxide dismutase-1 in CNS of transgenic amyotrophic lateral sclerosis models

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Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease afflicting the voluntary motor system. More than 100 different mutations in the ubiquitously expressed enzyme superoxide dismutase-1 (SOD1) have been associated with the disease. To search for the nature of the cytotoxicity of mutant SOD1s, amounts, enzymic activities and structural properties of the protein as well as the CNS histopathology were examined in multiple transgenic murine models. In order to generate the ALS phenotype within the short lifespan of the mouse, more than 20-fold increased rates of synthesis of mutant SOD1s appear to be required. The organs of transgenic mice expressing human wild-type SOD1 or either of the G93A and D90A mutant proteins showed high steady-state protein levels. The major proportion of these SOD1s in the CNS were inactive due to insufficient Cu charging and all contained subfractions with a reduced C57-C146 intrasubunit disulphide bond. Both G85R and the truncated G127insTGGG mutant showed low steady-state protein levels, lacked enzyme activity and had no C57-C146 disulphide bond. These mutants were also enriched in the CNS relative to other organs, suggesting inefficient recognition and degradation of misfolded disulphide-reduced SOD1 in susceptible tissues. In end-stage disease, despite 35-fold differences in levels of mutant SOD1s, similar amounts of detergent-resistant aggregates accumulated in the spinal cord. Small granular as well as larger more diffuse human SOD1 (hSOD1)-inclusions developed in all strains, the latter more pronounced in those with high hSOD1 levels. Widespread vacuolizations were seen in the strains with high levels of hSOD1 but not those with low, suggesting these alterations to be artefacts related to high hSOD1 levels and not to the ALS-causing cytotoxicity. The findings suggest that the motoneuron degeneration could be due to long-term exposure to misfolded aggregation-prone disulphide-reduced SOD1, which constitutes minute subfractions of the stable mutants and larger proportions of the unstable mutants.

Keywords: amyotrophic lateral sclerosis; superoxide dismutase; disulphide bond; misfolding; aggregates

Abbreviations: ALS = amyotrophic lateral sclerosis; CCS = copper chaperone for superoxide dismutase; G127X = Gly127insTGGG; SOD = superoxide dismutase; ww = wet weight

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Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative syndrome characterized by adult-onset progressive loss of motoneurons in the motor cortex, brainstem and spinal cord. About 10% of ALS cases are familial (Haverkamp et al., 1995) and in some of these the disease has been linked to mutations in the CuZn-superoxide dismutase (SOD1) gene (Rosen et al., 1993). Overall, ~5% of all cases with ALS show SOD1 mutations (Andersen et al., 2003). To date, 114 different SOD1 mutations have been identified in ALS patients, with all but one, D90A, showing a dominant mode of inheritance (Andersen et al., 2003). SOD1 is composed of two equal 153 amino acid subunits, each containing one Cu...
and one Zn ion. The enzyme catalyses the dismutation of the superoxide anion radical under formation of hydrogen peroxide: \(2\text{O}_2^- + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2\). The Cu ion carries out the catalysis, while the Zn ion stabilizes the structure of the subunits (Forman and Fridovich, 1973). Most mutant SOD1s show in patients reduction in enzyme activity in erythrocytes (Robberecht et al., 1994; Andersen et al., 1997), other cell types (Tsuda et al., 1994) and CNS tissue (Bowling et al., 1993). Even so, the disease is not caused by loss of function, since ALS patients homozygous for the D90A mutation show full SOD activity in both erythrocytes (Andersen et al., 1995) and in the CNS (Jonsson PA, Graffino SG, Brännstrom T, Andersen PM, Marklund SL, in preparation). Moreover, mice lacking SOD1 do not develop ALS (Reaume et al., 1996) and the SOD activity in the CNS is elevated in several of the multiple murine mutant SOD1 transgenic ALS models that have been generated (Gurney et al., 1994; Wong et al., 1995). The data suggest a cytotoxic gain of function conferred by the mutations. SOD1 is ubiquitously expressed, in many tissues at higher levels than in the brain and spinal cord (Marklund, 1984; Jonsson et al., 2004). Both the nature of the cytotoxicity and the reasons for the particular susceptibility of some parts of the CNS remain to be explained.

The different mutant SOD1 proteins are likely to cause ALS by essentially the same mechanism. Many transgenic murine ALS models have been generated in which mutant SOD1s of widely different characteristics are expressed. These provide an opportunity to search for patterns and common alterations that could help to elucidate the form of the SOD1 mutants that exert the noxious effects, as well as the reasons for the particular susceptibility of areas of the CNS harbouring the motor system. In this study, we examined biochemical and histopathological alterations in SOD1 in transgenic mice expressing the following mutations: D90A, G93A (Gurney et al., 1994), G85R (Bruijn et al., 1997) and G127insTGGG (G127X) (Jonsson et al., 2004). Mice overexpressing wild-type human SOD1 (hSOD1) and SOD1 knockouts served as references. The results suggest that ALS may be caused by cytotoxic, misfolded disulphide-reduced subfractions of the mutant enzymes which are enriched in the spinal cord and brain relative to other organs. The susceptibility of some areas of the CNS may be caused by their inability to recognize and degrade misfolded SOD1s rather than by the existence of tissue-specific vulnerable factors.

Materials and methods

Mice
The DNAs used for generation of transgenic mice expressing the G127X (Jonsson et al., 2004) and D90A (Jonsson PA, Graffino SG, Brännstrom T, Nilsson P, Andersen PM, Marklund SL, in preparation) mutants of hSOD1 were constructed in an 11.6 kb EcoRI–BamHI SOD1 genomic fragment (Levanon et al., 1985). The G127X mice used were homozygous line 716 mice and the D90A mice were homozygous line 134 mice. The construct for the G85R mice (Bruijn et al., 1997) was prepared in a 12 kb EcoRI–BamHI DNA fragment (Epstein et al., 1987), as were the constructs for G93A and wild-type hSOD1 (N1029) transgenic mice (Gurney et al., 1994). Two G93A lines were used, G93AGur (G1) and a line with reduced copy number, G93AGur\(\text{c}^\text{R}\). All the transgenic mice used were backcrossed 4–20 generations in C57Bl6 mice. C57Bl6 mice were used as controls. For comparison purposes, SOD1 knockout mice were obtained from A. Reaume (Reaume et al., 1996).

The mice were killed at preselected intervals, or when they were so terminally ill that they could not reach the food in their cages. Brain, spinal cord and peripheral organs were rapidly dissected out, frozen in liquid nitrogen and stored at –80°C. Other mice were perfusion-fixed with 4% paraformaldehyde in 0.1 M Na phosphate buffer, pH 7.4. The animal care and experiments were carried out in accordance with the European Communities Council Directive (86/609/EEC).

Recombinant hSOD1 variants
Recombinant hSOD1 variants were coexpressed with the copper chaperone for superoxide dismutase (CCS) in Escherichia coli and purified as previously described (Ahl et al., 2004). CuSO\(_4\) (3 mM) and ZnSO\(_4\) (30 \(\mu\)M) were added to the culture medium. The metal contents were determined by graphite furnace atomic absorption.

Cu-enriched diet
Following weaning at 21 days, G93AGur transgenic mice were put on a chow enriched with 400 p.p.m. Cu. Parallel litters were given ordinary chow. The mice were killed for analysis when terminally ill, at about 124 days of age.

The Cu contents of the spinal cords of control non-transgenic mice were determined by inductively coupled plasma atomic emission spectrometry using a PE Optima 3000XL apparatus (Perkin-Elmer, Boston, MA).

Northern blot
Total RNA was prepared from mouse brains using the Trizol reagent (Invitrogen, Carlsbad, CA) and the Northern blots were carried out as previously described (Jonsson et al., 2004). The samples were normalized against \(\beta\)-actin and the quantifications were carried out at least twice.

Homogenization of tissues
Generally, tissues were homogenized in 25 volumes of PBS (10 mM K phosphate, pH 7.0, in 0.15 M NaCl with EDTA-free Complete (Roche Diagnostics, Mannheim, Germany) anti-proteolytic cocktail added, using an Ultraturrax (IKA, Staufen, Germany) for 2 min. This was followed by sonication of the homogenate using a Sonifier Cell Disruptor (Branson, Danbury, CT) for 1 min. For the analysis of detergent-resistant aggregates, the PBS was supplemented with 0.1% NP40 (Roche Diagnostics, Mannheim, Germany) and in studies of the detergent of the intramolecular disulphide bond, 20 mM iodoacetamide was added to the buffer. For quantification of total tissue SOD1 and CCS content and activity and unless otherwise stated, a buffer containing 50 mM K phosphate, pH 7.4, 3 mM EDTA, 0.3 M KBr and Complete with EDTA was used instead.

Supplementation of homogenates with Cu
The tissues were homogenized in 25 volumes of PBS as described above. CuSO\(_4\) (1 mM) or an equal volume of PBS was added to the
homogenates. They were then incubated overnight at 4°C, followed by analysis of SOD activity. Three mice of each strain were analysed. Non-transgenic mice and SOD1 knockout mice were used as controls.

**Analysis of detergent-resistant aggregates**

Brain and spinal cord samples from the mice were homogenized in PBS (pH 7.0) with 0.1% of the detergent Nonidet P40 (NP40) added (Roche Diagnostics, Mannheim, Germany). The homogenized samples were then centrifuged at 20,000 g for 30 min at 4°C. The supernatants were removed and the pellets were resuspended and sonicated in double the original volume of homogenizing solution, followed by centrifugation. This washing step was repeated five times. Following the last wash, the pellets were resuspended and sonicated in 1 × SDS–PAGE sample buffer. The samples were then analysed by immunoblotting, using the anti-peptide 24–39 antibody.

**Antibodies**

Polyclonal rabbit antibodies were raised against keyhole limpet haemocyanin-coupled peptides corresponding to amino acids 4–20, 24–39 (human-specific), 43–57, 58–72, 80–96, 100–115 and 131–153 in the hSOD1 sequence. Mouse SOD1-specific antibodies were raised against a peptide corresponding to amino acids 24–36 of the mSOD1 sequence. Antibodies directed against CCS were raised against a peptide corresponding to amino acids 24–36 of the human CCS sequence. The antisera were affinity-purified in two steps, as previously described (Jonsson et al., 2004).

**Immunoblotting and quantification of SOD1**

The immunoblots were carried out as previously described (Jonsson et al., 2004). For quantification of hSOD1s by immunoblotting, the human-specific 24–39 antibody was used and wild-type hSOD1 with the concentration determined by quantitative amino acid analysis (Marklund et al., 1997) was used as original standard. For quantification of mSOD1, the mouse-specific 24–36 antibody was used. The mSOD1 was assumed to have the same specific activity as the human enzyme. In general, the quantifications by immunoblot were run at least in duplicate for each sample.

**Analysis of SOD activity**

SOD activity was determined by the direct spectrophotometric method using K$_2$O$_2$ (Marklund, 1976). One unit is defined as the SOD activity that brings about a decay of O$_2^-$ at a rate of 0.1 s$^{-1}$ in 3 ml buffer. One unit in the assay corresponds to 4.2 ng human wild-type and D90A mutant SOD1 (Marklund et al., 1997).

**Cytochrome oxidase**

Cytochrome oxidase was analysed in spinal cord homogenates solubilized with lauryl maltoside (Birch-Machin et al., 1994).

**Histopathology**

After formalin perfusion, fixation and paraffin embedding, tissue pieces from mice of different ages were sectioned. Immunohistochemical staining was carried out with the Ventana immunohistochemistry system using the anti-SOD1 peptide antibodies and anti-GFAP (Dako, Denmark).

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**Results**

**Overexpressed mutant hSOD1s have different molecular characteristics, but similar noxious effects**

The mutants of hSOD1 expressed in the different mouse strains cover a wide range of molecular, thermodynamic and functional properties. D90A is fully active both in human erythrocytes (Andersen et al., 1995) and in the CNS (Jonsson PA, Graffmo SG, Brännstrom T, Nilsson P, Andersen PM, Marklund SL, in preparation), suggesting high in vivo stability. G93A is intermediate stable, whereas G85R is more rapidly degraded in cell cultures (Borchelt et al., 1994). The truncation mutant G127X is at the extreme; it lacks a large part of the C-terminal sequence including a β-strand of the β-barrel (Jonsson et al., 2004). As a consequence, it is unlikely that G127X would adopt any native structure under physiological conditions. Since the noxious effects of mutant SOD1s show strong gene-dosage effects (Jonsson et al., 2004), we determined the levels of gene expression in all the strains used in this comparison study (Table 1). hSOD1 was most highly expressed in the G93AGur mice (here set to 100%) and these mice also showed the shortest survival time in our laboratory (124 days). The other transgenic mouse strains showed mRNA expression levels around half of that of G93AGur: D90A, 51%; G93AGur$^{dl}$, 50%; G85R, 43% and G127X, 63%. Their mean survival lengths were 407, 253, 345 and 250 days, respectively. Thus, despite their distinctly different thermodynamic and structural properties, the mutant hSOD1s have relatively similar cytotoxic effects on the spinal cord. The level of hSOD1 mRNA in the brain of the wild-type hSOD1 transgenic mice was 60% of that found in the G93AGur mice.

**Markedly different steady-state levels of hSOD1 protein in the transgenic mice**

Transgenic mice carrying the different hSOD1s showed widely different levels of hSOD1 protein in the spinal cord and brain (Fig. 1A and B). After 100 days of age, there were only small

**Table 1 Expression of human SOD1 mRNA in transgenic strains**

<table>
<thead>
<tr>
<th>Transgenic strain</th>
<th>Percentage of G93AGur ± SD</th>
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<tr>
<td>G93AGur</td>
<td>100%</td>
</tr>
<tr>
<td>Wild-type hSOD1</td>
<td>60 ± 4%</td>
</tr>
<tr>
<td>D90A</td>
<td>51 ± 8%</td>
</tr>
<tr>
<td>G93AGur$^{dl}$</td>
<td>50 ± 10%</td>
</tr>
<tr>
<td>G85R</td>
<td>43 ± 6%</td>
</tr>
<tr>
<td>G127X</td>
<td>63 ± 8%</td>
</tr>
</tbody>
</table>

mRNA was analysed by Northern blot using a purified PCR fragment, covering exons 1–3 of the hSOD1 sequence, as a probe. Samples were normalized against β-actin. The values are the means of three individual mice and are expressed as percentage of the values for G93A mice, which were run in parallel. Each mouse was quantified at least twice.
Fig. 1 Analysis of SOD1 protein and activity and CCS in murine tissue homogenates. (A and B) Time courses of levels of SOD1 protein in spinal cord and brain, respectively, of control and transgenic mice. In transgenic mice the levels of hSOD1 were analysed by quantitative immunoblot. In non-transgenic control mice, the mSOD1 activities were analysed and protein levels were calculated using the specific activity of hSOD1. Details are given in Materials and methods. For non-transgenic mice and the D90A, G127X and wild-type hSOD1 transgenic mice, the data represent means of analysis of three different mice and for the others, analysis of pools from three mice. (C) Western immunoblot of mSOD1 in spinal cord from 100-day-old non-transgenic and transgenic mice. (D) mSOD1 in different organs from 100-day-old non-transgenic and hSOD1 transgenic mice, the data represent means of analysis of three different mice and for the others, analysis of pools from three mice. (E) hSOD1 levels in tissues of 100-day-old transgenic mice. Protein levels were determined by immunoblot, or calculated from SOD1 activities and the specific activity of hSOD1. The figure also shows such data following overnight incubation at 4°C with 1 mM CuSO4. (F) Murine CCS levels in spinal cords from hSOD1 transgenic mice, non-transgenic control mice and SOD1 null mice.
Disulphide-reduced mutant SOD1 enriched in the CNS

Brain and spinal cord than in peripheral organs. The low-level mutants G85R and G127X are higher in brain than in other tissues (Fig. 1D). Even more striking, the steady-state levels of mSOD1 as compared to other tissues (Fig. 1A). In control mice, the spinal cord and brain contain low levels of mSOD1, whereas only 90% and 45% of the mSOD1 level. Except for G93AGur, the hSOD1s are synthesized at similar rates in the mice according to the levels of mRNA (Table 1). Thus, the different steady-state contents thus reflect widely different in vivo stabilities and degradation rates of the various hSOD1s.

The level of endogenous mSOD1 is not influenced by transgenic overexpression of hSOD1s

The high degree of hSOD1 expression in the transgenic models could conceivably influence the turnover of mSOD1. In G127X, G85R, G93AGur, G93AGur dl and wild-type hSOD1 transgenic mice, however, there was no significant difference in mSOD1 content in spinal cord (Fig. 1C), brain, liver and kidney (not shown). The exception to the rule was the (strain 134) D90A mice, in which the mSOD1 levels were 50–60% of the controls in spinal cord, brain, liver and kidney. To explore the reasons for this result, another D90A transgenic strain, 154, with a mean survival length of 480 days, was also examined (Jonsson PA, Graffmo SG, Brännstrom T, Nilsson P, Andersen PM, Marklund SL, in preparation). In these mice, there were no reductions in mSOD1 content in the spinal cord (Fig. 1C) or in other organs. This suggests that the location of the transgene insertion in the 134 strain causes reduced transcription of mSOD1.

There are low amounts of SOD1 in the CNS compared to other tissues and inversion in mice carrying unstable mutants

In control mice, the spinal cord and brain contain low levels of mSOD1 as compared to other tissues (Fig. 1D). Liver and kidney contain 8- and 3-fold more, respectively, whereas only skeletal muscle contains less. Similar relationships regarding SOD1 content between tissues have been found in humans (Marklund, 1984). In the transgenic mice with high levels of hSOD1, however, the relative differences in hSOD1 content between brain and spinal cord, kidney and liver are clearly smaller (Fig. 1D). Even more striking, the steady-state levels of the low-level mutants G85R and G127X are higher in brain and spinal cord than in peripheral organs.

Insufficient Cu-charging of highly expressed hSOD1s in the CNS of transgenic mice

The truncated G127X protein lacks SOD activity (Jonsson et al., 2004). We have also found, as reported previously (Bruijin et al., 1997; Jonsson et al., 2004), that the G85R hSOD1 protein appears to lack activity in transgenic mice. The SOD1 activity in spinal cords of G85R transgenic mice was different from that in control mice [11 200 ± 1 060 (SD) U/g wet weight (ww) as opposed to 11 020 ± 1 170 (SD) U/g ww; both n = 11]. We analysed the activity of an E.coli-produced recombinant G85R hSOD1 preparation that was 11% Cu-charged and completely charged with Zn. Related to the Cu-content, the SOD activity of this preparation was 93% of that of a recombinant wild-type hSOD1 preparation. Thus, Cu-charged G85R hSOD1 shows full SOD activity. The lack of activity in the transgenic mice suggests that the G85R mutant protein is unable to retain Cu in competition with other ligands in vivo in mice. The G93AGur mice showed very high SOD1 activities, but the levels in brains and spinal cords—calculated from the SOD1 activity measurements and the specific activity of Cu-charged hSOD1 (Marklund et al., 1997)—were only 71 and 37%, respectively, of the protein levels measured in quantitative immunoblots (Fig. 1E). No such discrepancies were seen for kidney and liver. Likewise, in spinal cords from G93AGur dl, D90A and wild-type hSOD1 transgenic mice, the overexpressed hSOD1s proteins appeared to be only 65, 21 and 21% active (Fig. 1E).

In order to find an explanation for the discrepancies, three G93A brain and spinal cord extracts were incubated with 1 mM CuSO4 overnight. This increased the SOD1 activities of the brain extracts from 121 700 ± 14 200 to 183 800 ± 14 900 (SD) U/g ww and of the spinal cord extracts from 94 600 ± 8 700 to 195 800 ± 13 200 (SD) U/g ww, respectively. These SOD activities almost corresponded to the total SOD1 protein levels (Fig. 1E). Likewise, Cu-incubations of spinal cord extracts from the other high-level hSOD1 transgenic mice resulted in large increases in the SOD activities (Fig. 1E). The increases recorded were not due to unspecific effects of Cu in the SOD assay; 1 mM Cu reduced the total SOD activity of a spinal cord extract of a SOD1 null mouse from 800 to 190 U/g ww. The treatment thus appeared to partially inactivate the SOD2 activity. The SOD1 activities of spinal cord extracts from three control mice rose by 7%, from 11 200 ± 1 400 to 12 000 ± 600 (SD) U/g ww. Finally, Cu-incubation increased the activity of three G85R spinal cord extracts from 13 100 ± 3 300 to 17 700 ± 5 200 (SD) U/g ww. Subtracting the increase seen in the non-transgenic control mice, the G85R-related 28% increase corresponds to a 30% activity of the G85R protein.

The Cu content of spinal cords from non-transgenic control mice was 3.5 ± 1.05 (SD, n = 5) µg/g ww. To Cu-charge all hSOD1 in the spinal cords of 100-day-old wild-type hSOD1 transgenic mice, 6.3 µg Cu per g ww would be needed. Thus, to charge the hSOD1s in the bodies of transgenic mice,
much Cu is required. To determine whether the incomplete Cu-charging of hSOD1 could be caused by an insufficiency in dietary availability of Cu, G93AGur mice were kept on diets containing 400 p.p.m. Cu from weaning until terminal ill. There were no significant differences in SOD1 activities in spinal cords between these mice [127 600 ± 18 500 (SD) U/g ww, n = 5] and mice on a normal diet [121 300 ± 9 300 (SD) U/g ww, n = 4]. Supplementation with Cu did not influence the survival time of the mice.

Cytochrome oxidase, like SOD1, is charged via chaperones that derive Cu mainly from Ctr-1 in the plasma membrane (Valentine and Gralla, 1997). The activity of cytochrome oxidase is a measure of the Cu availability in tissues. The cytochrome oxidase activities in spinal cord extracts from the transgenic mouse strains (3–9 mice of each strain) were not significantly altered; all were within ±15% of the levels in controls (data not shown). This result provides evidence that the deficient Cu charging of SOD1 is not caused by a general insufficiency in the Cu uptake of the tissue.

To examine the final step, the Cu-charging of SOD1 by CCS (Culotta et al., 1997), the levels of murine CCS were analysed by immunoblotting of spinal cord extracts from 100-day-old mice (Fig. 1F). CCS was found to vary with the steady-state levels of SOD1. In mSOD1 knockout mice, the CCS levels were below half of those found in control mice. In G85R mice, the CCS levels were doubled and they were 25% higher in G127X mice. Finally, in the high-level mice, the G93AGur, G93AGur, D90A or wild-type hSOD1 transgenics, the CCS levels were 3–5-fold higher than in non-transgenic control mice. These increases are far less than the 8–24-fold increases in hSOD1 protein, suggesting that CCS may be limiting with regard to Cu-charging in the transgenic mice. This idea is supported by the fact that the largest proportion of active SOD1 (65%) is found in the strain with least hSOD1 protein, G93AGur.

**Significant fractions of both human and murine SOD1 carry a reduced intrasubunit disulphide bond in transgenic mice**

Human SOD1 contains four cysteines (C6, C57, C111 and C146). Two of these, C57 and C146, form an intrasubunit disulphide bond which links the flexible Zn-binding loop to β-strand 8 in the central β-barrel of the SOD1 subunit. Reductional cleavage of the disulphide bond substantially weakens the dimeric interaction and leads to monomerization of the subunits in the absence of metals (Arnesano et al., 2004). The integrity of the disulphide bond is also critical for the monomeric state. In its absence, the proportion of denatured and potentially aggregation-prone species is increased.

To study the status of the disulphide bond, fresh tissues from transgenic mice were homogenized in buffer containing 20 mM iodoacetamide to react with and block free thiol groups in proteins and other compounds. When such homogenates were subjected to SDS-PAGE and immunoblot analysis in the absence of reductant in the sample buffer, two bands were seen (Fig. 2A). These bands appeared to differ by 2–3 kDa in molecular weight. The upper band is assigned to SOD1 with the disulphide bond reduced, since all SOD1 protein appears at that position if the sample is reduced by mercaptoethanol prior to electrophoresis (Fig. 2A). Analogous patterns were seen in homogenates treated with another thiol-blocker, N-ethylmaleimide (not shown). The separation in the gel could thus arise from the linearized disulphide-reduced SOD1 being more retarded in the matrix than the corresponding species restricted by the disulphide bond. To further confirm the assignment of the bands, recombinant mutant hSOD1s coexpressed with CCS in E.coli (Ahl et al., 2004) were used as reference materials (Fig. 2B). Consistently, the wild-type recombinant hSOD1 showed mobility similar to that of the disulphide-oxidized band in a G93AGur spinal cord homogenate. Also, the control mutant hSOD1 with all four cysteines mutated to alanines (CallA) had mobility similar to that of the disulphide-reduced band in the brain homogenate. The hSOD1 with only Cys6 and Cys111 mutated to alanines behaved as if it were disulphide-oxidized. In reducing gels, the SOD1s in the G93A spinal cord and the reference samples showed identical mobilities (Fig. 2B). Similar mobility differences have been reported for *in vivo* disulphide-reduced and oxidized forms of a prokaryotic SOD1 (Battistoni et al., 1999) and *in vitro* reduced and oxidized recombinant hSOD1s (Furukawa and O’Halloran, 2005).

The proportion of disulphide-reduced SOD1 in a sample not treated with reductant can be determined by comparison with a standard curve created from dilutions of reduced tissue extract (Fig. 2A). From this comparison, it becomes obvious that the disulphide-oxidized subunit has a lower antigenic reactivity than the reduced subunit in the blot. The proportion of disulphide-reduced hSOD1 in the G93A spinal cord extract in Fig. 2A was ~6%. It is important that the thiol blocker iodoacetamide is present in the homogenates to prevent artificial oxidative formation of the disulphide bond. In its absence the disulphide-reduced band becomes weaker with a half-life of 2 h in homogenates kept at room temperature (not shown).

Disulphide-reduced hSOD1 was analysed in spinal cord, brain, kidney and liver in the different transgenic mouse strains and was detected in all cases (Table 2). Throughout, the proportions were nearly equal in brain and spinal cord and lower in kidney and liver. The proportion of disulphide-reduced hSOD1 did not change significantly with the age of the mice (Fig. 2C). No disulphide-oxidized band could be discerned in the G85R (Fig. 2D) and G127X mice. To ascertain that disulphide-oxidized and disulphide-reduced G85R hSOD1 can be differentiated from each other in the blotting assay, G85R in a spinal cord extract was compared with a recombinant G85R preparation produced in *E.coli* (Fig. 2E). The recombinant preparation showed a higher mobility in the non-reducing gel and was apparently all in disulphide-oxidized form. Upon reduction of the samples...
by mercaptoethanol, both showed the same mobility as the G85R from spinal cord in the non-reducing gel. From the standard curves, the recoveries of reduced G85R and G127X (which, owing to the C-terminal truncation, cannot be disulphide oxidized) were both around 50%. This can be explained by the presence of multiple bands of higher molecular weight in the non-reduced sample, which disappear in the presence of mercaptoethanol (Fig. 2D). These bands most
Fig. 3E. Despite the very large differences in steady-state levels although with a slower time course.

spinal cord extracts from wild-type hSOD1 transgenic mice, a similar increase in detergent-resistant aggregates was seen in detergent-resistant hSOD1 aggregates (Fig. 3A–C). Strikingly, transgenic mice with terminal disease contained much less et al. in the final pellets, together with heavy widespread smearing.

phase of the disease. In all cases, bands at 30–40 kDa were seen in the spinal cords of all transgenic mouse strains. The percentage of disulphide-reduced hSOD1 in tissues from 100-day-old mice of different transgenic strains was determined as outlined in Fig. 2A. Mean values for three mice in the G93A Gur group and for two mice in the others, are given. For G85R and G127X, the amount of disulphide-reduced hSOD1 appeared to be around 50 according to the standard curve. This discrepancy appears to be largely accounted for by the presence of multiple bands of high molecular weight in the non-reduced lane (Fig. 2D).

The disulphide-oxidation state of the mSOD1 was also examined, using the mSOD1-specific antibody (Fig. 2F). No disulphide-reduced band could be discerned in control mice, or in G85R and G127X transgenic mice. On the other hand, in the G93A Gur, D90A and wild-type hSOD1 transgenic mice, the proportions of disulphide-reduced mSOD1 appeared to be similar to the proportions of reduced hSOD1 in the same extracts. The time courses of disulphide-reduced mutant human and endogenous murine SOD1 in spinal cords were similar in G93A transgenic mice (Fig. 2C).

Table 2 Proportion of disulphide-reduced hSOD1 in different transgenic mouse strains

<table>
<thead>
<tr>
<th></th>
<th>Spinal cord (%)</th>
<th>Brain (%)</th>
<th>Liver (%)</th>
<th>Kidney (%)</th>
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<tbody>
<tr>
<td>G93AGur</td>
<td>8</td>
<td>5</td>
<td>1.9</td>
<td>3</td>
</tr>
<tr>
<td>Wild-type hSOD1</td>
<td>14</td>
<td>13</td>
<td>1.4</td>
<td>6</td>
</tr>
<tr>
<td>D90A</td>
<td>14</td>
<td>14</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>G85R</td>
<td>100</td>
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<td>G127X</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
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</table>

The table shows the percentage of disulphide-reduced hSOD1 in different transgenic mouse strains. The highest proportion was seen in G93AGur, followed by wild-type hSOD1, D90A, G85R, and G127X.

In all mouse strains, there was more background SOD1 staining in motoneurons than in other cell types. This staining increased with time and it was, as expected, stronger in the mice with high levels of hSOD1, than in mice with low levels (Fig. 4A, B, D, E, G, H, J, K, M, N, P and Q). With time, this staining condensed to larger more homogenous inclusions, with a terminal surge in occurrence. These inclusions were clearly more abundant in the mice with high levels of hSOD1 (Fig. 4E, H and K). This condensation of hSOD1 affects motoneurons to different degrees and at later time points one can in one section see motoneurons with almost normal appearance, while other motoneurons contain many inclusions. There were also small dense granular SOD1-immunoreactive inclusions in motoneurons and the neuropil (Fig. 4B, D, E, G, H, J, K, N and Q). The neuropil inclusions may exist in the astrocytic and dendritic compartments, or they may represent phagocytosed remnants of degenerate cells. These small granular inclusions were the alteration that differed least between the transgenic ALS models and they thus form a common denominator.

SOD1-immunoreactive axons were seen in the ventral roots in the high-level strains (Fig. 4C, F and L) but not in the low-level strains (Fig. 4O and R). In the high-level transgenic mice, there were also marked vacuolizations in ventral funiculi and roots and in most of the vacuoles there was a rim of SOD1 positivity. No such alterations were seen in the low-level transgenic mice.

Likewise, there were distinct progressive vacuolizations with rim staining in the neuropil of the high-level transgenic mice (Fig. 4B, E and K), but not in G85R and G127X mice (Fig. 4N and Q). The similarities suggest that the neuropil alterations mainly represent pathology in axons and axon collaterals. The absence of such changes in the low-level transgenic mice suggests that the vacuolization axonal pathology is an artifact associated with extreme levels of hSOD1 protein in some of the models and it is possibly not relevant to ALS.

Smaller vacuoles could also be discerned in motoneuron somata of the high-level transgenic mice (Fig. 4B, E and K).

Histopathology

The time course of histopathological alterations in spinal cords was examined in transgenic mice. The mice formed two groups with distinctly different patterns of morphological tissue reaction. The mouse strains with low levels of hSOD1, G85R and G127X, could be grouped together in terms of histopathological changes, as could the mice with high levels, i.e. D90A, G93AGur and G93AGur61. The wild-type hSOD1 transgenic mice were similar to the latter group, but with less advanced changes and delayed occurrence. The motoneuron loss did not differ to any obvious degree between the different ALS models studied. In all strains of mice, an appreciable number of cells persisted even at terminal stages.

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probably represent hSOD1 disulphide-coupled to other SOD1 molecules or other proteins, which suggests that (misfolded disulphide-reduced) hSOD1 easily engages in such reactions. They could also indicate increased oxidant stress in the tissue (Cumming et al., 2004). The proportions of the higher-molecular-weight bands were lower in the transgenic mutant mice with high levels of hSOD1 protein (not shown).

The disulphide-oxidation state of the mSOD1 was also examined, using the mSOD1-specific antibody (Fig. 2F). No disulphide-reduced band could be discerned in control mice, or in G85R and G127X transgenic mice. On the other hand, in the G93A Gur, D90A and wild-type hSOD1 transgenic mice, the proportions of disulphide-reduced mSOD1 appeared to be similar to the proportions of reduced hSOD1 in the same extracts. The time courses of disulphide-reduced mutant human and endogenous murine SOD1 in spinal cords were similar in G93A transgenic mice (Fig. 2C).

Detergent-resistant hSOD1 aggregates increase with time in the spinal cords of all transgenic mouse strains.

Spinal cord homogenates were extracted five times with the detergent NP40 and the final pellets were analysed by immunoblotting (Fig. 3A–E). Pelleted hSOD1 was seen at all times, but with a distinct accumulation at the terminal phase of the disease. In all cases, bands at 30–40 kDa were seen in the final pellets, together with heavy widespread smearing. Similar findings have been reported previously in some models (Johnston et al., 2000; Shinder et al., 2001; Wang et al., 2002; Jonsson et al., 2004). In comparison, the brains of transgenic mice with terminal disease contained much less detergent-resistant hSOD1 aggregates (Fig. 3A–C). Strikingly, a similar increase in detergent-resistant aggregates was seen in spinal cord extracts from wild-type hSOD1 transgenic mice, although with a slower time course.

Immunoblots of detergent-resistant hSOD1 material from equal amounts of terminal spinal cord extracts are shown in Fig. 3E. Despite the very large differences in steady-state levels of the enzyme (Fig. 1A), the total amounts of hSOD1 in aggregates in terminal spinal cords were relatively similar (Fig. 3E).
but not in G85R and G127X mice (Fig. 4N and Q). These probably represent swollen mitochondria (Wong et al., 1995; Jaarsma et al., 2001) and as with the axonal changes, are apparently related to the occurrence of extreme levels of hSOD1 protein.

In all models, there was a time-dependent increase in the number and staining intensity of glial fibres as judged from GFAP immunocytochemistry (not shown). There was no significant difference between the different strains of mice expressing mutant hSOD1s. A weaker and delayed reaction was seen in the transgenic mice expressing wild-type hSOD1.

Discussion

Estimation of SOD1 turnover

The levels of hSOD1 in the spinal cords of wild-type hSOD1 transgenic mice were 24-fold higher than the endogenous levels of mSOD1 in control mice (Fig. 1A). No significant changes in the background levels of mSOD1 were observed (Fig. 1C). There was thus no overload of the systems that degrade mSOD1 as a result of high-level expression of hSOD1. The murine and human wild-type SOD1s should have similar rates of turnover in the spinal cords of transgenic mice. If this is the case, the rate of synthesis of wild-type hSOD1 is about 24-fold higher than that of the endogenous mSOD1. Comparison of the relative mRNA levels (Table 1) suggests that the rates of synthesis of hSOD1 in the transgenic strains vary between 17 (G85R) and 40 times (G93AGur) the background synthesis rate of mSOD1. Thus, very high synthesis rates of mutant SOD1s appear to be necessary for expression of ALS-like phenotypes within the short lifespan of mice. There is a strong inversely proportional gene dosage effect. In two lines of G127X mice, the survival times were almost twice as long in hemizygous mice as in homozygous
mice for the insertions (Jonsson et al., 2004). The G93AGur<sup>dl</sup> mice, which showed mRNA levels that were 50% of those of G93AGur mice, survived 253 days as compared to 124 days. Broadly, a 25-fold increased rate of mutant hSOD1 synthesis appears to cause disease within a year in the murine models. By simple inference from the data of this study, one can predict that humans heterozygous for SOD1 mutations (50% of the control synthesis rate), would develop terminal
disulphide-reduced mutant SOD1 enriched in the CNS

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Disease at 25/0.5 × 1 year = 50 years of age, as is actually the case (Andersen et al., 2003).

Cytotoxic and cytoidal effects of mutant SOD1s have been demonstrated in many in vitro cell culture studies. The expression levels of the mutant SOD1s are in general only a few times higher than the background endogenous SOD1 levels in the cells and the noxious effects are usually observed within days. The susceptible areas of the CNS of mice are thus much more resistant to mutant SOD1s than cultured cells, suggesting that the noxious mechanisms could differ between the in vivo situation and the in vitro cell culture studies.

A limitation of the present study is that whole tissue was analysed, while primarily motoneurons are injured in the transgenic models. The cell type(s) in which mutant SOD1s exert their noxious effects, however, is/are unknown. Specific expression of mutant SOD1s in astrocytes (Gong et al., 2000), neurons in general (Pramatarova et al., 2001) and motoneurons (Lino et al., 2002) has failed to induce motoneuron disease in mice. Studies in chimeric mice (Clement et al., 2003) as well as in co-cultures of glial and neuronal cells (Ferri et al., 2004) have indicated that the simultaneous presence of mutant hSOD1 in several cell types causes a tissue reaction that results in motoneuron injury. These latter studies support the validity of the present approach using tissue analysis.

Accumulation of hSOD1 aggregates is a hallmark of the terminal phase of ALS associated with mutant SOD1s

In the spinal cords of terminally ill mice, all the mutant hSOD1s developed detergent-resistant aggregates of multiple molecular forms, with heavy smearing (Fig. 3A–C and E). An almost identical picture was seen in spinal cord ventral horns, but not elsewhere in the CNS, in a patient carrying the G127X mutation (Jonsson et al., 2004). Numerous studies have indicated early-commencing, long-term noxious effects of mutant SOD1s in mice (for references see Jonsson et al., 2004), which suggests that the terminal aggregates appear too late to be major participants in the pathogenesis. Importantly, however, they constitute a least common denominator for all the transgenic models (Fig. 3A–C, E and 4) (Johnston et al., 2000; Shinder et al., 2001; Wang et al., 2002; Jonsson et al., 2004) and for patients carrying SOD1 mutations (Jonsson et al., 2004). The aggregates can possibly be regarded as terminal markers of the long-term assault from cytotoxic misfolded SOD1.

Is the oxidative formation of the C57-C146 disulphide bond linked to Cu-charging?

SOD1 species with a reduced disulphide bond existed in all transgenic strains and G85R hSOD1 appeared completely reduced. The endogenous mSOD1 in the transgenic mice was found to mimic the disulphide bond reduction of the G93A, D90A and wild-type, but not the G127X and G85R hSOD1s (Fig. 2C and F). This suggests that the incomplete oxidation of the disulphide bond is related to insufficient Cu-charging in the high-level mouse strains, which should also pertain to the mSOD1. The mechanisms by which structural disulphide bonds can be formed in the strongly reducing cytosol are not understood (Rietsch and Beckwith, 1998). The present data indicate that formation and maintenance of the disulphide bond in SOD1 are at least partially linked to the Cu-charging and possibly the CCS itself, as has also been suggested by studies in yeast (Brown et al., 2004; Furukawa et al., 2004). However, since the major part of the inactive SOD1 is disulphide-oxidized, the oxidation cannot be compulsorily linked to the Cu-charging.

Model for ALS-causing toxicity of SOD1

The 114 mutant SOD1s associated with ALS most likely cause the disease by the same mechanism. The compositions of hSODs in spinal cords were found to be complex and to vary a great deal among the various transgenic strains (Fig. 5). A consideration of the data suggests that the damage is caused by minute amounts of a common cytotoxic hSOD1 species. The results also suggest that this noxious form is disulphide-reduced. Reduced subunits would probably adopt cytotoxic properties more easily than their disulphide-oxidized counterparts, because of their higher configurational freedom and lower stability (Lindberg et al., 2004). The ALS-associated C146R mutant and six C-terminal SOD1 truncation mutants (Andersen et al., 2003), which for structural reasons permanently lack the disulphide bond, support this notion; the disulphide bond is not required for provocation of the disease. Even so, it is clear that all disulphide-reduced species of hSOD1 populating the cells cannot be cytotoxic. The steady-state levels of disulphide-reduced hSOD1 are highest in wild-type hSOD1 transgenic mice. At the same time, wild-type hSOD1 has proved to be less susceptible to disulphide reduction in vitro than mutants associated with ALS (Tiwari and Hayward, 2003). This suggests that the reduced wild-type enzyme is generally more stable in vivo than the reduced mutants. This conclusion is also consistent with the results of thermodynamic (Lindberg et al., 2005) and melting point (Furukawa and O’Halloran, 2005) analyses of wild-type and mutant hSOD1s in vitro.

An additional factor that would be expected to modulate protein stability in vivo is the binding of Zn and Cu. Ligation of Zn compensates for the loss of stability caused by reduction of the disulphide bond and promotes retention of the dimeric state (Arnesano et al., 2004). Thus, different abilities to coordinate Zn could affect the steady-state levels of the reduced protein, possibly favouring the wild-type protein over the mutants. The Zn-binding affinities (Crow et al., 1997) and also the metal ion specificities (Goto et al., 2000) are significantly weakened in the ALS-associated hSOD1 mutants tested. Consistent with the idea that the metal-binding ability is indeed a critical determinant of protein turnover in vivo, the truncated variant G127X and also G85R (which appears to be unable to retain Cu in vivo), both show low overall...
**Fig. 5** Amounts of different molecular forms of hSOD1 in spinal cords from 100-day-old mice of different transgenic lines. The columns were calculated from the data in Fig. 1A and E and Table 2. In the high-competition situation for Cu, the disulphide-reduced subfractions of G93A, D90A and wild-type hSOD1 were presumed to be inactive. The mSOD1 in non-transgenic control mice is presented as a striped column.

**Fig. 6** Hypothetical mechanism of formation of noxious forms of hSOD1 in the spinal cord. The various mutant hSOD1 proteins fold and mature to different extents, giving rise to multiple molecular forms after synthesis (cf. Fig. 5). A selection of the many possible variants is depicted in the illustration. The wild-type hSOD1 and stable mutants should be degraded in larger proportions via non-selective routes such as autophagy to lysosomes. More unstable and hence short-lived, mutants unfold to greater extents and are recognized by the quality-control systems for proteasomal degradation. Unfolded/misfolded disulphide-reduced forms of hSOD1 show higher steady-state levels in the spinal cord and brain than in peripheral organs, suggesting slow recognition and degradation in the CNS (Fig. 1D). These forms and possibly also oligomeric protoaggregates, may exert cytotoxic effects which injure the motor areas of the CNS.
steady-state hSOD1 levels in tissues of transgenic mice (Figs 1A, B, D and 5). The putative cytotoxic forms of SOD1 may constitute large proportions of these and minute subfractions of the high-level mutants G93A and D90A.

Following synthesis, the mutant hSOD1s form native metal-charged dimers and many other structural variants (Figs 5 and 6). The degradation routes of stable cytosolic proteins in the CNS are not well understood, but as in other organs, they may proceed partially via non-selective autophagy of the cytosol and lysosomal lysis (Yoshimori, 2004). SOD1 degradation by such non-selective pathways occurs in the liver (Rabouille et al., 1993) and this could be an innocuous high-capacity route of mutant and wild-type SOD1 turnover in the CNS. The action of such non-selective high-capacity degradation would explain the lack of influence of high hSOD1 expression on the turnover of mSOD1 (Fig. 1C). The more stable the SOD1 variant, the greater the proportion that is degraded via a non-selective route.

The ALS-linked hSOD1 mutants are conformationally less stable than the wild-type enzyme and have an increased propensity to unfold (and possibly also to misfold) in vivo. Thus, they are also more likely to be recognized and degraded via the quality control systems and the proteasome. The complete unfolding of the hSOD1 structure occurs subsequent to dissociation of the homodimer and is promoted by reductive cleavage of the C57–C146 disulphide bond (Lindberg et al., 2004). The enrichment of G85R and G127X hSOD1 proteins in the CNS (Fig. 1D) suggests that the quality control and degradation systems for reduced and misfolded hSOD1s are less efficient in the brain and spinal cord than in other organs. Such unfolded subfractions of mutant hSOD1s could be responsible for the toxic effects, e.g. by interaction with essential factors such as the antiapoptotic protein Bcl-2 (Pasinelli et al., 2004), by accumulating in mitochondrial outer membranes (Liu et al., 2004), or by forming oligomeric protoaggregates which are commonly believed to cause cytotoxicity (Bucciantini et al., 2002; Volles and Lansbury, 2002; Walsh et al., 2002).

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