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Inducible Superoxide Dismutase 1 Aggregation in Transgenic Amyotrophic Lateral Sclerosis Mouse Fibroblasts

Bradley J. Turner, Elizabeth C. Lopes, and Surindar S. Cheema*

Howard Florey Institute of Experimental Physiology and Medicine, University of Melbourne, Victoria, Australia

Abstract High molecular weight detergent-insoluble complexes of superoxide dismutase 1 (SOD1) enzyme are a biochemical abnormality associated with mutant SOD1-linked familial amyotrophic lateral sclerosis (FALS). In the present study, SOD1 protein from spinal cords of transgenic FALS mice was fractionated according to solubility in saline, zwitterionic, non-ionic or anionic detergents. Both endogenous mouse SOD1 and mutant human SOD1 were least soluble in SDS, followed by NP-40 and CHAPS, with an eight-fold greater detergent resistance of mutant protein overall. Importantly, high molecular weight mutant SOD1 complexes were isolated with SDS-extraction only. To reproduce SOD1 aggregate pathology in vitro, primary fibroblasts were isolated and cultured from neonatal transgenic FALS mice. Fibroblasts expressed abundant mutant SOD1 without spontaneous aggregation over time with passage. Proteasomal inhibition of cultures using lactacystin induced dose-dependent aggregation and increased the SDS-insoluble fraction of mutant SOD1, but not endogenous SOD1. In contrast, paraquat-mediated superoxide stress in fibroblasts promoted aggregation of endogenous SOD1, but not mutant SOD1. Treatment of cultures with peroxynitrite or the copper chelator diethyldithiocarbamate (DDC) alone did not modulate aggregation. However, DDC inhibited lactacystin-induced mutant SOD1 aggregation in transgenic fibroblasts, while exogenous copper slightly augmented aggregation. These data suggest that SOD1 aggregates may derive from proteasomal or oxidation-mediated oligomerisation pathways from mutant and endogenous subunits respectively. Furthermore, these pathways may be affected by copper availability. We propose that non-neural cultures such as these transgenic fibroblasts with inducible SOD1 aggregation may be useful for rapid screening of compounds with anti-aggregation potential in FALS. J. Cell. Biochem. 91: 1074–1084, 2004.

Key words: aggregation; amyotrophic lateral sclerosis; DDC; fibroblasts; oxidant stress; proteasome; SOD1

Abnormal protein misfolding, oligomerisation and deposition is associated with selective neuronal death in late-onset neurodegenerative disease [Taylor et al., 2002; Soto, 2003, reviews]. Intracellular aggregated superoxide dismutase 1 (SOD1) is a neuropathologic feature of human familial amyotrophic lateral sclerosis (FALS) [Shibata et al., 1996] linked to mutations in the *SOD1* gene [Deng et al., 1993; Rosen et al., 1993]. Transgenic overexpression of different human SOD1 missense or truncation mutants in mice, including $SOD1^{A4V/WT}$ [Deng et al., 2001], $SOD1^{G37R}$ [Wong et al., 1995], $SOD1^{H46R}$ [Nagai et al., 2000], $SOD1^{H46R/H48Q}$ [Wang et al., 2002a], $SOD1^{L84V}$ [Kato et al., 2001], $SOD1^{G85R}$ [Bruijn et al., 1997], $mSOD1^{G86R}$ [Ripps et al., 1995], $SOD1^{D90A}$ [Brannstrom et al., 1998], $SOD1^{G93A}$ [Gurney et al., 1994], $SOD1^{I113T}$ [Kikugawa et al., 2000], $SOD1^{L126X}$ [Deng et al., 1999], $SOD1^{G127X}$ [Jonsson et al., 2004] and the recent artificial Quad mutant $SOD1^{H46R/H48Q/H63G/H120G}$ [Wang et al., 2003], induces motor neuron degeneration invariably with protein aggregate pathology. Ubiquitinated SOD1-containing neurofilamentous and Lewy body-like hyaline inclusions (LBHI) have been identified in both spinal cord motor neurons and astrocytes of disease-expressing

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*Correspondence to: Assoc. Prof. Surindar S. Cheema, Howard Florey Institute of Experimental Physiology and Medicine, University of Melbourne, Victoria 3010, Australia. E-mail: s.cheema@hfi.unimelb.edu.au

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mice [Bruijn et al., 1997]. These histological structures are preceded by small biochemically discrete detergent-insoluble complexes of mutant SOD1 detected months before disease-onset [Johnston et al., 2000; Wang et al., 2002]. Recently, fibrillar and amyloid-like assemblies were detected in oligomeric and structurally destabilised SOD1 mutants [DiDonato et al., 2003; Elam et al., 2003], suggesting a potential pathogenic role for such fibrous aggregates in FALS. The age-dependent and nervous system accumulation of SOD1 aggregates in transgenic mice [Wang et al., 2002] also supports a causal relationship between abnormal SOD1 deposition and motor neuron degeneration in FALS.

Intracellular SOD1 aggregates have been reproduced in culture by transfection of primary neurons and a variety of cell lines. Selective aggregation and toxicity of mutant SOD1 in motor neurons, but not sensory or hippocampal neurons, was first demonstrated in primary cultures [Durham et al., 1997]. SOD1 aggregates have since been generated in Neuro2A and PC12 cells [Lee et al., 2002; Takeuchi et al., 2002], in addition to non-neural COS7, HEK 293 and NIH 3T3 cells expressing SOD1 mutations [Hoffman et al., 1996; Koide et al., 1998; Bruening et al., 1999; Johnston et al., 2000]. Lastly, SOD1 aggregates were also induced in a novel organotypic spinal cord slice model prepared from transgenic FALS mice [Puttaparthi et al., 2003]. The manipulation and modulation of SOD1 aggregation in vitro clearly has direct implications for human and animal models of FALS. For instance, the upregulation of stress-inducible hsp70 or hsp40 reduced mutant SOD1 aggregate burden and improved viability of cultures [Bruening et al., 1999; Takeuchi et al., 2002], supporting the role of protein misfolding in mutant SOD1-mediated toxicity. Similarly, agents that promote SOD1 aggregation in vitro may be informative about FALS etiopathogenesis.

Here, we report that primary transgenic fibroblast cultures isolated from the $SOD1^{G93A}$ mouse model of FALS demonstrate protein aggregate and solubility changes characteristic of spinal cord in disease. Both proteasomal inhibition and oxidative stress induced SOD1 aggregation in fibroblasts, as modulated by copper availability, implicating these processes in nervous system accumulation of SOD1 in FALS.

MATERIALS AND METHODS

Chemicals

Nonidet P-40 (NP-40), diethyldithiocarbamate (DDC), $CuCl_2$, $ZnCl_2$, EDTA, lactacystin, paraquat, minocycline, protease inhibitor cocktail and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (Castle Hill, New South Wales, Australia). 3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate (CHAPS) was obtained from Calbiochem (Croydon, Victoria, Australia). Sodium dodecyl sulfate (SDS) was purchased from Astral Scientific (Gymea, New South Wales, Australia). Peroxynitrite was obtained from Upstate Biotechnology (Parkville, Victoria, Australia). Target Vorpal Bunny-1 (TVB-1) was synthesised by K. Macfarlane (School of Chemistry, Monash University, Victoria, Australia) by reacting *o*-anisidine with potassium iodide, ethylbromoacetate and proton sponge in dry acetonitrile under nitrogen gas overnight followed by column chromatography purification.

Transgenic Mice

Transgenic $SOD1^{G93A}$ mice derived from the B6SJL-TgN(SOD1-G93A)1Gur line (The Jackson Laboratory, Bar Harbor, ME) were bred on a B6SJL background by mating transgenic males with non-transgenic females. Animals were PCR-genotyped using DNA extracted from tail biopsies [Gurney et al., 1994]. For procedures, newborns were anaesthetised by ice-induced hypothermia and promptly decapitated. Adult mice were killed by intraperitoneal injection of Lethabarb (200 mg/kg) (Lyppard Victoria, Cheltenham, Australia). These experiments were performed in accordance with the guidelines set by the Monash University Animal Ethics and Experimentation Committee (permit number: BAM/A/2001/4).

Sequential Protein Extraction

Lumbar spinal cord segments from 90-day-old transgenic $SOD1^{G93A}$ or wild-type mice ($n = 3$ of each genotype) were dissected, snap frozen and homogenised in liquid nitrogen-chilled mortars. Powdered cords were resuspended in 500 μl of Tris-buffered saline (TBS)-extraction buffer containing 10 mM Tris-HCl, pH 7.4, 170 mM NaCl, 5 mM EDTA and 1% (v/v) protease inhibitor cocktail on ice for 15 min and centrifuged at 15,800*g* for 15 min at

4°C to collect saline-soluble supernatants. Protein pellets were sequentially solubilised with increasing concentrations of NP-40, CHAPS or SDS dissolved in TBS-extraction buffer [range 0.5–10% (v/v)] and detergent-soluble supernatants were progressively collected. Solubilisation cycles involved washing pellets twice in TBS-extraction buffer, resuspending in detergent on ice for 15 min, centrifugation at 15,800*g* for 15 min at 4°C and collection of supernatants. Procedures were performed at room temperature for cycles involving ≥2% SDS content due to crystallisation effects. The protein concentration of supernatants was quantified using the bicinchoninic acid (BCA) assay based on bovine serum albumin standards (Promega, Annandale, New South Wales, Australia).

Primary Fibroblast Derivation

Neonatal fibroblasts were prepared using modified procedures [Huang et al., 1997]. The dorsal trunk skin from newborn transgenic $SOD1^{G93A}$ or wild-type mice (n = 4 of each genotype) was dissected, ethanol sterilised, cleaned of muscle and immersed in 1 ml of sterile mouse tonicity phosphate-buffered saline (MT-PBS), pH 7.4. The tissue was dissociated by finely chopping with a sterile razor blade and passing through an 18-gauge needle in sterile MT-PBS. The cell suspension was transferred to 100 mm culture dishes containing Dulbecco's modified Eagles medium (DMEM) (Invitrogen, Mulgrave, Victoria, Australia) supplemented with 10% (v/v) foetal calf serum (FCS) and 1% (v/v) penicillin–streptomycin, and incubated at 37°C for a week. Dermal explants were removed, adherent cells rinsed in MT-PBS and cultured to confluency before passaging to 75 cm^2 flasks. Fibroblasts were split 1:2 every 3–4 days and expanded to a maximum of eight passages for assays. The viability of fibroblasts (passage 2) was measured by 2 h treatment with MTT (0.5 mg/ml), followed by cell solubilisation in DMSO and absorbance readings at 530 nm.

Immunocytochemistry

Fibroblasts were plated at 1×10^4 cells per 1.5 mm plastic coverslip (Nunc, Rochester, NY) in 1 ml culture medium in 24-well dishes for 48 h. Cells were fixed in 4% (w/v) paraformaldehyde in MT-PBS for 20 min and endogenous peroxidase activity quenched by incubation in 3% (v/v) H_2O_2 in MT-PBS for 10 min. Cells were permeabilised with 0.4% (v/v) TX-100 for 10 min, incubated with blocking solution of 5% (v/v) horse serum and 5% (w/v) skim milk in MT-PBS for 1 h, and stained overnight with a human/mouse reactive SOD1 antibody (1:200, Calbiochem, Alexandria, New South Wales, Australia). Cells were rinsed three times in MT-PBS, incubated with biotinylated antibodies (1:200, Vector Laboratories, Burlingame, CA) for 1 h then HRP-conjugated streptavidin-biotin (1:100) for 1 h. Staining was visualised by applying a solution of 0.05% (w/v) 3,3-diaminobenzidine (DAKO, Botany, New South Wales, Australia) and 0.05% (v/v) H_2O_2 in MT-PBS for 5 min. Cells were post-fixed in 0.01% (w/v) osmium tetroxide in MT-PBS for 1 min then visualised and photographed with an Olympus AX70 microscope.

Aggregation Assays

Fibroblasts were plated at 5×10^3 cells per well in 100 μl culture medium in microwell dishes. The medium was replaced 4 h later with 0–10 μM freshly prepared lactacystin, paraquat, peroxynitrite or DDC in medium and cells treated overnight (16 h). In separate experiments, 10 μM lactacystin was co-administered with 0.1 μM $CuCl_2$, $ZnCl_2$, DDC, EDTA, TVB-1, paraquat, peroxynitrite or minocycline (non-toxic doses) and cells treated overnight. At the conclusion of drug treatments, culture medium from microwells was centrifuged at 5,000*g* for 5 min and supernatant fractions assayed for lactate dehydrogenase (LDH) activity (Roche, Nunawading, Victoria, Australia) as evidence of cytotoxicity. Fibroblasts were then lysed in 50 mM Tris-HCl buffer, pH 7.5, with 150 mM NaCl, 2 mM EDTA, 1% (v/v) NP-40, 1% (v/v) TX-100 and 1% (v/v) protease inhibitors for 10 min on ice and extracted with an equal volume of 2× reducing SDS-loading buffer before boiling for 5 min.

For sequential protein extraction, confluent fibroblast cultures (2×10^6 cells) in 75 cm^2 flasks were treated overnight with 10 μM lactacystin. Cells were rinsed twice with MT-PBS, scraped in 1 ml TBS-extraction buffer and sequentially extracted using SDS as stated above for spinal cord tissues.

Western Blotting

Spinal cord proteins (5 μg per lane) and whole fibroblast lysates were separated using 12.5% SDS–polyacrylamide gels and transferred to nitrocellulose filters (Schleicher and Schuell,

Dassel, Germany), 0.2 μm pore size. Membranes were blocked with 5% (w/v) defatted milk, 0.1% (v/v) Tween-20 and 0.02% (w/v) NaN_3 in TBS for 1 h then incubated overnight with the SOD1 antibody (1:1,000). Blots were washed three times in TBS-Tween-20 (TBST) for 10 min, incubated with HRP-conjugated antibodies (1:2,500, Chemicon, Boronia, Victoria, Australia) for 1 h, washed four times in TBST for 10 min and developed using enhanced chemiluminescence (ECL) reagents (Roche). TBS, NP-40, CHAPS and SDS-soluble proteins were transferred, stained and detected with hyperfilm (Amersham-Pharmacia, Baulkham Hills, New South Wales, Australia) using identical exposure times. Scanning densitometry of protein bands was performed using Scion Image software (version 4.0.2, Scion Corporation, Frederick, MD) whereby the area under the curve defined by two minima was measured and expressed as fold-variation. Bands were quantified from three independent experiments.

Statistical Analysis

Cell viability and cytotoxicity data were compared using one-way ANOVA with Tukey's post hoc test using GraphPad Prism software (version 3.02, GraphPad Software, Inc., San Diego, CA).

RESULTS

SDS-Insoluble Mutant SOD1 Predominates in Transgenic Spinal Cord

To examine the effects of various detergents on SOD1 protein solubility, lumbar spinal cords from transgenic $SOD1^{G93A}$ and wild-type mice were homogenised and fractionated according to solubility in saline (TBS), zwitterionic (CHAPS), non-ionic (NP-40) or anionic (SDS) detergents. Detergent-insoluble pellets were sequentially solubilised with increasing detergent strength, and the resulting soluble supernatants were serially collected and analysed by Western blotting for SOD1 content. The elimination of SOD1 from a fraction was used as an index of relative solubility between detergents. Thus, the differential solubility of mutant human SOD1 and endogenous mouse SOD1 was quantified using this assay.

In wild-type mouse spinal cord, the majority of endogenous SOD1 was TBS-soluble (Fig. 1). The TBS-insoluble endogenous SOD1 fractions were abolished by 0.5% NP-40, 0.5% CHAPS and 2% SDS, suggesting reduced solubility in SDS. In contrast, mutant SOD1 extracted from age-matched transgenic spinal cords was highly resistant to solubilisation in all three detergents (Fig. 2). The TBS-insoluble mutant SOD1

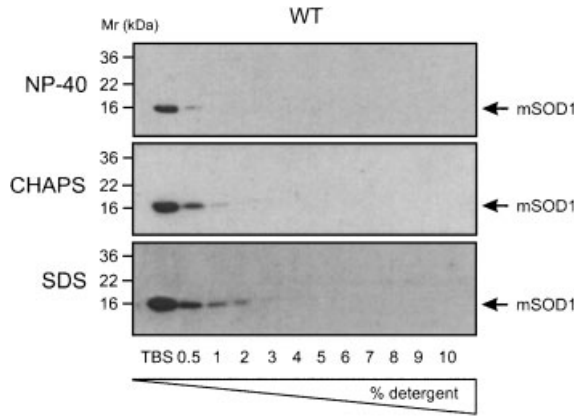

Fig. 1. Differential detergent solubility of endogenous mouse superoxide dismutase 1 (mSOD1) from wild-type mice. Postnatal day 90 mouse spinal cords were fractionated according to solubility using a sequential biochemical extraction in saline (TBS) and increasing concentrations of non-ionic (NP-40), zwitterionic (CHAPS) or anionic (SDS) detergent. Detergent-soluble fractions were resolved by SDS–PAGE, electroblotted and measured for SOD1 content using a human/mouse reactive antibody. The 16 kDa endogenous SOD1 is least soluble in SDS.

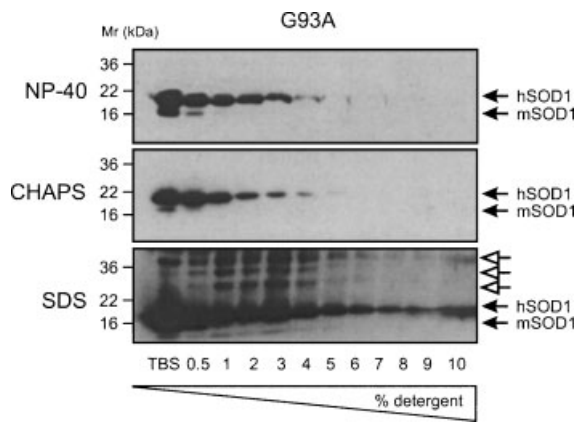

Fig. 2. Differential detergent solubility of mutant human SOD1 (hSOD1) and endogenous mouse SOD1 (mSOD1) from transgenic familial amyotrophic lateral sclerosis (FALS) mice. Postnatal day 90 mouse spinal cords were fractionated according to solubility using a sequential biochemical extraction in saline (TBS) and increasing concentrations of non-ionic (NP-40), zwitterionic (CHAPS) or anionic (SDS) detergent. Detergent-soluble fractions were resolved by SDS–PAGE, electroblotted and measured for SOD1 content using a human/mouse reactive antibody. The 22 kDa mutant SOD1 is poorly soluble in SDS detergent and precipitates as high molecular weight oligomers (open arrows).

fractions were eliminated by 4% NP-40 and 4% CHAPS, representing an eight-fold enhancement in detergent resistance compared to endogenous SOD1. However, mutant SOD1 resisted complete solubilisation in SDS, even at the maximum detergent strength tested of 10%. More importantly, high molecular weight SOD1 complexes were resolved in SDS-soluble fractions, but not NP-40 or CHAPS-extracts (Fig. 2). These oligomers were dissociated completely by 6% SDS. The solubility index of endogenous SOD1 co-expressed with mutant SOD1 was not altered in transgenics compared to wild-type animals (Fig. 1).

Transgenic Fibroblasts Express Mutant SOD1 Without Intrinsic Aggregate Burden

To investigate the solubility and aggregation of SOD1 proteins using an in vitro expression system, neonatal primary fibroblasts were harvested and cultured from wild-type and transgenic mice. The expression of SOD1 was characterised by immunocytochemical and Western analysis of cultures. SOD1 was present in wild-type fibroblasts as a diffuse cytoplasmic staining with little nuclear localisation (Fig. 3a). In contrast, an intense perinuclear distribution of SOD1 was observed in transgenic fibroblasts, appearing granular and fibrillar upon higher magnification. Immunoblot analysis of wild-type fibroblasts revealed a single ~16 kDa band corresponding to endogenous SOD1 (Fig. 3b). Transgenic fibroblasts expressed both endogenous SOD1 and a more abundant ~22 kDa species representing mutant SOD1, without apparent high molecular weight oligomers. The level of mutant SOD1 protein was approximately four-fold greater than endogenous SOD1 in wild-type fibroblasts by scanning densitometry (Fig. 3c). Since mutant SOD1 aggregation is a postnatal phenomenon, lysates from serially passaged fibroblasts over 1 month were analysed. The absence of aggregates over time with passage (Fig. 3b) suggests that transgenic fibroblasts do not inherently accumulate SOD1 complexes. To assess the effect of mutant SOD1 expression on cell viability, MTT reduction was measured in fibroblasts (Fig. 3d). A significant decline in viability was determined in transgenic fibroblasts compared to wild-type cells ($P < 0.05$), suggesting that oxidative but not aggregate burden implicated in FALS is reproduced by these cells.

Proteasomal Inhibition by Lactacystin Promotes Mutant SOD1 Aggregation in Transgenic Fibroblasts

To stimulate SOD1 aggregation in fibroblasts, cells were treated overnight with the irreversible proteasome inhibitor lactacystin. Exposure of wild-type fibroblasts to lactacystin promoted some accumulation, but not aggregation of endogenous SOD1 at 1–10 μM concentrations (Fig. 4a). Remarkably, lactacystin-induced a large dose-dependent accumulation and aggregation of mutant SOD1 in transgenic fibroblasts, with discrete high molecular weight oligomers appearing with 10 μM treatment (Fig. 4a). To examine the effect of SOD1 aggregates on the indices of detergent solubility measured previously on spinal cords, lactacystin-treated and untreated transgenic fibroblasts were sequentially extracted with SDS. Proteasomal inhibition of fibroblasts increased the SDS-insoluble fraction of mutant SOD1 compared to untreated cells (Fig. 4b). A three-fold enhancement in SDS-resistance from 2 to 6% detergent was achieved by overnight lactacystin treatment.

Paraquat-Mediated Superoxide Stress Promotes Endogenous SOD1 Aggregation in Wild-type Fibroblasts

To assess the effects of oxidative agents on SOD1 aggregation, fibroblasts were treated overnight with freshly prepared paraquat, peroxynitrite or DDC. Exposure of cultures to paraquat induced oligomerisation of endogenous SOD1 in wild-type fibroblasts but not endogenous or mutant SOD1 in transgenic cells (Fig. 5a). A single high molecular weight oligomer appeared in wild-type fibroblasts treated with the maximum dose of paraquat at 10 μM only. Peroxynitrite or DDC alone did not influence SOD1 aggregation (Fig. 5a). Assay of LDH release by cultures after treatment revealed significant toxicity produced by paraquat in wild-type fibroblasts ($P < 0.05$), but not mutant cells (Fig. 5b), while DDC and peroxynitrite were not lethal.

Copper-Chelation Inhibits Lactacystin-Induced Mutant SOD1 Aggregation in Transgenic Fibroblasts

Lastly, the effects of redox metals, metal chelators, oxidants and neuroprotective agents on inducible mutant SOD1 aggregation by

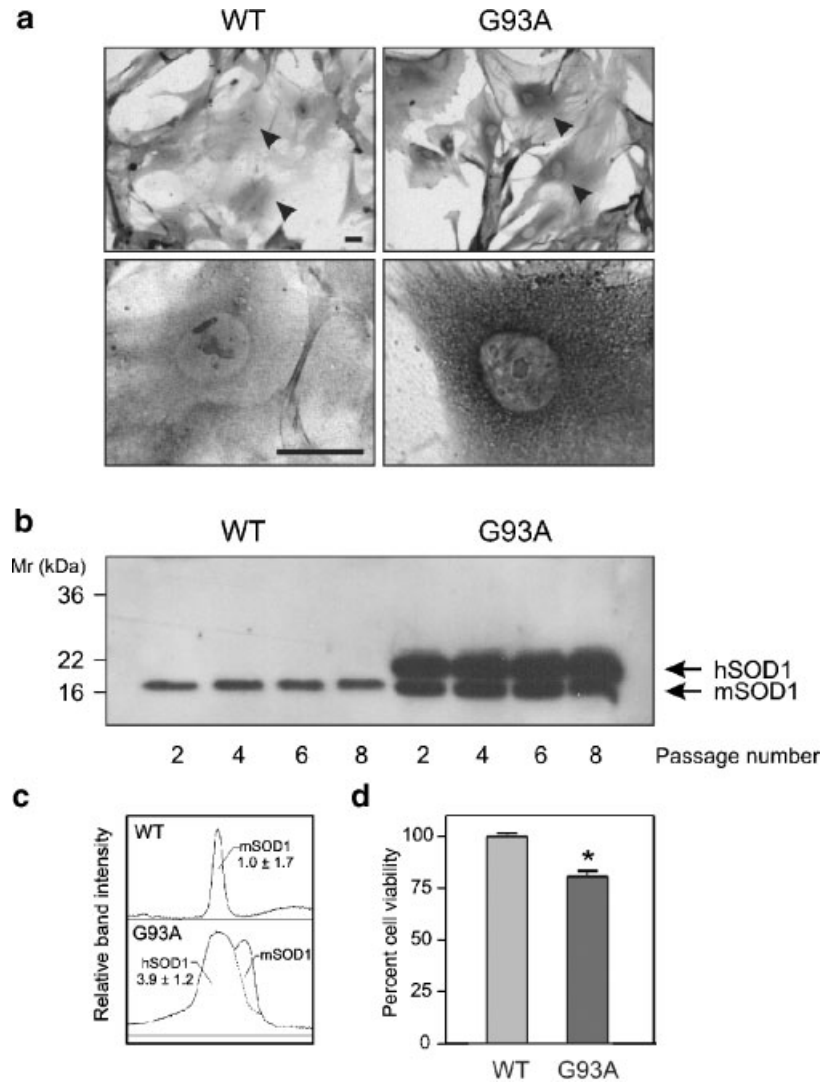

Fig. 3. Expression of SOD1 proteins by neonatal transgenic fibroblasts. Primary fibroblasts were isolated and cultured from newborn SOD1^{G93A} and wild-type mice. **a**: Immunocytochemical analysis of SOD1 expression and localisation in cells at 48 h reveals an intense perinuclear distribution in transgenic, but not wild-type fibroblasts (arrowheads); scale bar, 10 μm. **b**: Immunoblot analysis of serially passaged cells reveals an absence of SOD1 aggregates over time. **c**: Scanning densitometry of SOD1 protein expression by fibroblasts (mean ± SEM). **d**: Cell viability of cultures at 24 h assessed by MTT reduction shows a deficit in transgenic fibroblasts. Values represent means ± SEM of triplicate wells, $^*P < 0.05$ difference from wild-type cells.

proteasomal inhibition was tested on transgenic fibroblasts. The presence of calcium chelators (EDTA, TVB-1), paraquat, peroxynitrite and minocycline did not modulate lactacystin-mediated aggregation (Fig. 6). However, the dual copper chelator and SOD1 inhibitor, DDC, abolished aggregate formation. Treatment with zinc also reduced aggregate burden. Conversely, exposure to copper slightly increased the intensity of SOD1 complexes compared to lactacystin-treated alone cells.

DISCUSSION

These experiments describe the first account of mutant SOD1 biochemical abnormalities in non-neural primary cells derived from transgenic FALS mice. Despite differences in cellular physiology to motor neurons, these isolated fibroblasts maintained the relative expression level of endogenous to mutant SOD1 protein observed in mouse neural tissues [Gurney et al., 1994], and exhibited protein aggregation and

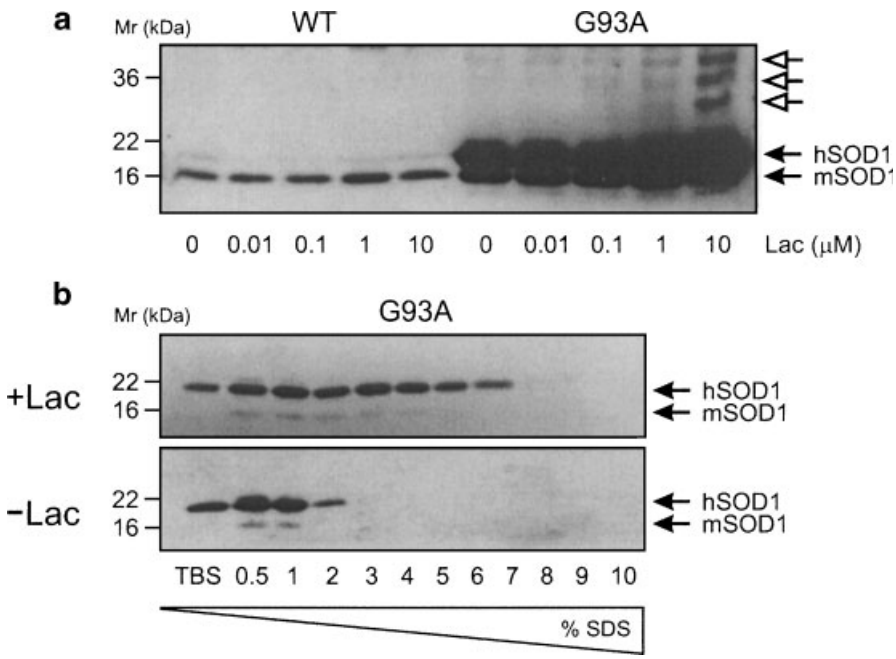

Fig. 4. Induction of hSOD1 accumulation and aggregation by lactacystin treatment of mutant fibroblasts. **a**: Immunoblot analysis of SOD1 proteins in fibroblasts treated with lactacystin (Lac) for 16 h. High molecular weight mutant SOD1 oligomers (open arrows) are present in treated transgenic fibroblasts but not wild-type cells. **b**: Differential solubility of mutant SOD1 from lactacystin treated (+) and untreated (−) fibroblasts. Cells were fractionated according to solubility using a sequential biochemical extraction in saline (TBS) and increasing concentrations of anionic (SDS) detergent. Detergent-soluble fractions were resolved by SDS–PAGE, electroblotted and measured for SOD1 content using a human-mouse reactive antibody. Inhibition of the proteasome increases the SDS-resistant burden of mutant SOD1.

solubility changes characteristic of FALS. The utility of this fibroblast expression system for investigation of SOD1 aggregation mechanisms was therefore demonstrated.

Intraneuronal protein insolubility is a hallmark feature of many neurodegenerative disorders [Johnson, 2000, review]. We first fractionated SOD1 proteins according to saline and detergent solubility to determine mutant-specific changes in spinal cords of transgenic FALS mice. A similar strategy was previously used to identify disease-associated changes in α-synuclein [Campbell et al., 2001]. Three detergents with varying solubility strengths were tested; NP-40, a weak non-ionic detergent disrupting plasma but not nuclear membranes; CHAPS, a moderate zwitterionic detergent with improved solubility; and SDS, a strong ionic detergent and denaturant disrupting protein hydrophobic interactions. Mutant SOD1 was least soluble in all three detergents compared to endogenous SOD1, with SDS-insoluble fractions persisting in spinal cord tissue even at high detergent concentrations. Importantly, SDS-resistant complexes of mutant SOD1 but not NP-40 or CHAPS-resistant oligomers were detected following extraction. Taken together, these data suggest that resistance to solubilisation and dissociation by anionic detergent, specifically, is an acquired property of misfolded $SOD1^{G93A}$ mutants. Although few biochemical and biophysical properties of mutant SOD1 correlate with FALS progression [Ratovitski et al., 1999], altered detergent solubility indicative of increased aggregation propensity remains a consistent feature of cell lines, spinal cord cultures and transgenic mice expressing SOD1 mutations [Shinder et al., 2001; Wang et al., 2003].

In transgenic fibroblasts, proteasomal inhibition was necessary to induce mutant SOD1 aggregation and insolubility. The failure of fibroblasts to spontaneously develop aggregates may reflect an absence of extrinsic cytoplasmic factors or declining function common to degenerating motor neurons [Wang et al., 2002]. Alternatively, mutant SOD1 aggregation may be limited to postnatal or adult cells since transgenic neonatal mouse spinal cords do not assemble SOD1 complexes [Turner et al., 2003],

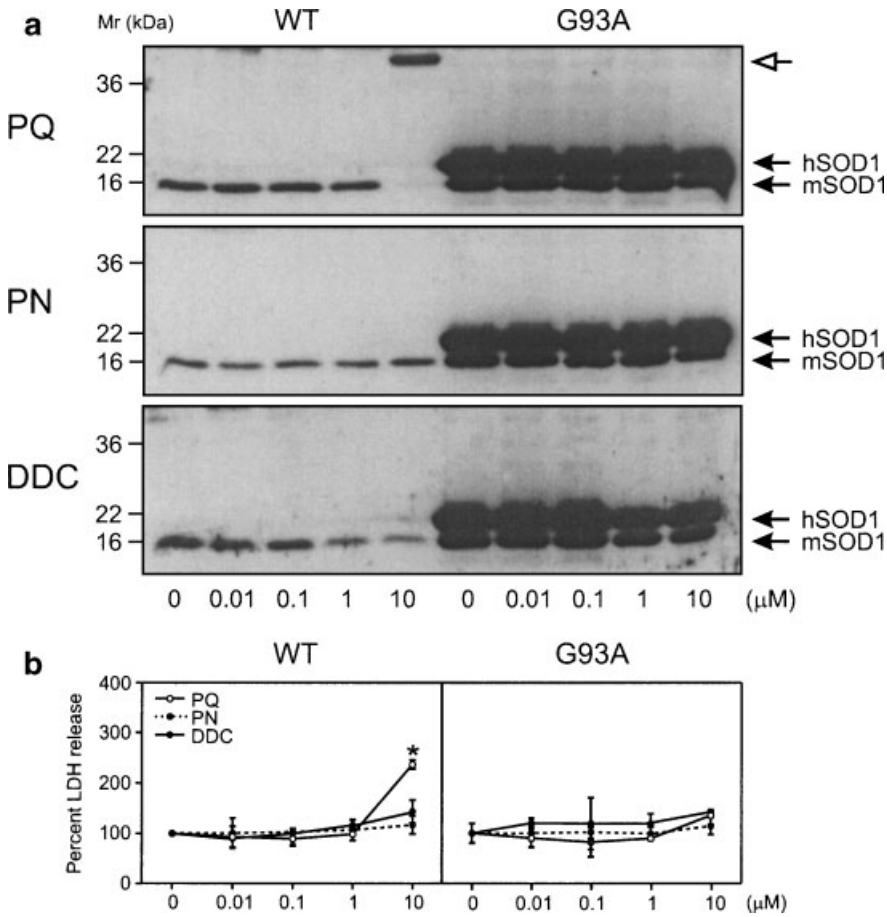

Fig. 5. Induction of mSOD1 aggregation and toxicity by paraquat in wild-type fibroblasts. **a**: Immunoblot analysis of SOD1 proteins in fibroblasts exposed to paraquat (PQ), peroxynitrite (PN) and diethyldithiocarbamate (DDC) for 16 h. Endogenous SOD1 oligomers (open arrow) appear in paraquat-treated wild-type cells but not transgenic fibroblasts. **b**: Cytotoxicity assay of treated cultures determined by LDH release. Values represent means ± SEM of triplicate wells. Paraquat induces selective toxicity in wild-type fibroblasts, $^*P < 0.05$ difference from untreated cells.

nor do organotypic spinal cord slice cultures prepared from early postnatal day transgenic mice in the absence of proteasomal inhibitors [Puttaparthi et al., 2003]. The ubiquitin and ATP-dependent proteasome represents a major pathway for degradation and clearance of mutant SOD1 [Hoffman et al., 1996]. Consequently, inhibition of the proteasome enhances the stability of mutant SOD1 leading to accumulation [Hoffman et al., 1996; Johnston et al., 2000]. In this study, lactacystin-mediated blockade of the proteasome increased both wild-type and mutant SOD1, leading to selective aggregation of mutant SOD1 only. A decline in proteasome activity and expression was reported in ageing rat spinal cord [Keller et al., 2000], suggesting a role of the proteasome in late-onset neurodegenerative disease. Recently, proteasomal function was progressively impaired in a mutant SOD1 expressing neural cell line [Urushitani et al., 2002]. Thus mutant SOD1 aggregates may represent both a consequence and cause of proteasomal dysfunction, eventually causing motor neuron death.

Challenge of fibroblasts with acute oxidative stress induced endogenous SOD1 oligomers in wild-type cells only. Interestingly, aggregation correlated with cytotoxicity at the highest paraquat dose, suggesting that SOD1 oligomerisation may manifest a cellular response or defence to oxidant stress [Lee et al., 2002]. This all-or-nothing band shift observed with SOD1 has been previously reported in studies of prion protein (PrP) treated with EDTA [Wadsworth et al., 1999]. Increasing evidence suggests that intramolecular aggregation is a property of both mutant and wild-type SOD1 under certain circumstances [Lee et al., 2002; Urushitani

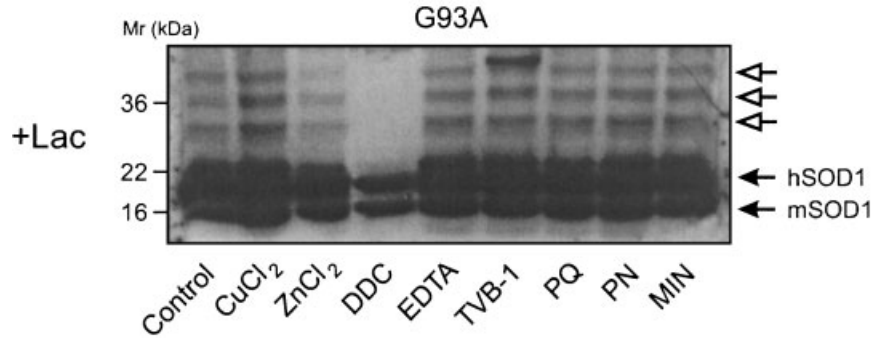

Fig. 6. Modulation of lactacystin-mediated mutant human SOD1 (hSOD1) aggregation in mutant fibroblasts. Immunoblot analysis of SOD1 proteins in fibroblasts treated with 10 μM lactacystin with redox metals ($CuCl_2$, $ZnCl_2$), metal chelators (DDC, EDTA, TVB-1), oxidants (PQ, PN) or minocycline, all at 0.1 μM, for 16 h. Lactacystin-induced mutant SOD1 aggregation is inhibited largely by DDC.

et al., 2002]. When reacted with singlet oxygen or copper-ascorbate, purified wild-type SOD1 assumed aggregated forms [Kim et al., 2001; Rakhit et al., 2002]. Treatment of SOD1-transfected cells with hydrogen peroxide also induced wild-type aggregates [Urushitani et al., 2002]. Finally, C-terminally truncated peptides of wild-type SOD1 showed increased aggregation potential in vitro [Wang et al., 2003]. We now confirm that the superoxide donor paraquat mediates wild-type SOD1 oligomerisation in fibroblasts. These results suggest that native substrate–enzyme interactions, SOD1-associated peroxidative activity [Wiedau-Pazos et al., 1996] or exogenous oxidants may induce SOD1 misfolding and aggregation, without a familial mutation. The presence of aggregated SOD1 in cases of non-SOD1-linked SALS supports this idea [Shibata et al., 1994].

Transgenic fibroblasts were resistant to paraquat toxicity in this study. This contrasts with previous reports that fibroblasts obtained from FALS patients showed increased sensitivity to oxidative stress compared with control cells [Aguirre et al., 1998]. We determined a four-fold increase in mutant SOD1 expression relative to endogenous SOD1 in fibroblasts, consistent with brain levels of SOD1 in transgenic mice [Gurney et al., 1994]. We suggest that overexpression of mutant SOD1 in fibroblasts, with the full complement of $SOD1^{G93A}$ specific activity, has short-term antioxidant benefits in culture. Thus, transgenic cells are protected against paraquat toxicity and endogenous SOD1 aggregation, unlike wild-type fibroblasts expressing endogenous enzyme alone. However, long term accumulation of mutant SOD1 in motor neurons in vivo with age-related oxidant stress and proteasomal impairment [Keller et al., 2000] may prove deleterious.

The copper chelator DDC inhibited the formation of mutant SOD1 complexes induced by lactacystin treatment. DDC is a potent inhibitor of SOD1 dismutase and peroxidase activities, shown previously to protect cultured cells from mutant SOD1-induced apoptosis [Wiedau-Pazos et al., 1996]. Although administration of DDC failed to delay disease in $SOD1^{G93A}$ mice [Heiman-Patterson et al., 2001], the success of other compounds such as D-penicillamine and iron porphyrins in extending survival [Hottinger et al., 1997; Wu et al., 2003] supports a role for chelation therapy in FALS. Our data suggest that DDC interferes with mutant SOD1 aggregation directly, since metal ions regulate protein conformation linked to neurodegeneration [Wadsworth et al., 1999], or indirectly via activation of the proteasome [Nothwang et al., 1992]. In contrast to copper chelation by DDC, treatment of fibroblasts with copper ions increased mutant SOD1 complexes while zinc decreased aggregates. The role of zinc in mutant SOD1 pathogenesis is uncertain, since both dietary zinc supplementation or restriction accelerates death in $SOD1^{G93A}$ mice [Ermilov et al., 2002; Groeneveld et al., 2003].

In conclusion, this study supports a role of proteasomal dysfunction and pro-oxidative conditions in the genesis of aggregated SOD1, whether mutated or wild-type enzyme. Furthermore, the propensity for SOD1 aggregation may already reside in the wild-type enzyme. Induction of the proteasome and antioxidant defences in vivo could therefore be rational

approaches to reducing SOD1 aggregate burden, if indeed essential to pathogenesis in FALS.

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