Expression of a Cu,Zn superoxide dismutase typical of familial amyotrophic lateral sclerosis induces mitochondrial alteration and increase of cytosolic Ca^{2+} concentration in transfected neuroblastoma SH-SY5Y cells

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Abstract We have set up a model system for familial amyotrophic lateral sclerosis (FALS) by transfecting human neuroblastoma cell line SH-SY5Y with plasmids directing constitutive expression of either wild-type human Cu,Zn superoxide dismutase (Cu,ZnSOD) or a mutant of this enzyme (G93A) associated with FALS. We have tested mitochondrial function and determined cytosolic Ca^{2+} concentration in control cells (untransfected) and in cells expressing either wild-type Cu,ZnSOD or G93A. We report that G93A induces a significant loss of mitochondrial membrane potential, an increased sensitivity toward valinomycin and a parallel increase in cytosolic Ca^{2+} concentration. The above phenomena are not related to total Cu,ZnSOD content and activity in the cell.

Key words: Superoxide dismutase; Amyotrophic lateral sclerosis; Mitochondrial membrane potential; Cytosolic calcium; Oxidative damage; Neurodegeneration

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a progressive, lethal disease characterized by degeneration of cortical and spinal motoneurons. 120 years after Charcot's original description, many of the mechanisms underlying this pathology are still poorly understood.

ALS occurs both sporadically (SALS) and as a familial, age-dependent autosomal dominant disorder; more than 50 point mutations in the gene encoding human Cu,Zn superoxide dismutase (Cu,ZnSOD) have been reported to be responsible for familial amyotrophic lateral sclerosis (FALS) [1,2]. Most autosomal-dominant FALS cases are indistinguishable from SALS on the basis of clinical and pathologic criteria [3]. This similarity suggests that sporadic and familial forms share similar pathogenetic mechanisms. However, little is known about those mechanisms which would explain how the mutant SODs might result in motoneuron injury. Present evidence indicates that ALS is a multifactorial disease [4] in which selective loss of motoneurons reflects a complex interplay among oxidative injury, excitotoxic stimulation of the motoneuron and dysfunction of critical proteins such as neurofilaments [5]. Furthermore, failure of calcium homeostasis seems to play a central role among the mechanisms presumably involved in the pathogenesis of ALS; low levels of Ca^{2+} binding proteins have been detected in human motoneurons [6,7]; increased Ca^{2+} concentration is present in ALS patients [7] and many sporadic ALS patients possess serum antibodies to both L-type and P-type calcium channels [4,8] which are able to increase calcium entry in a motoneuron cell line [9]. Mitochondrial damage seems also to be involved, since alterations of these organelles have been reported in transgenic mice overexpressing mutant SOD [10] and changes in the activities of the mitochondrial electron transport chain complex I have been reported in FALS patients [3].

In order to investigate whether such cellular alterations might be associated with the presence of mutated Cu,ZnSODs in vivo, we have undertaken the construction of a cellular model system. Mutation G93A, one of those found in FALS families, has been introduced in the cDNA encoding human Cu,ZnSOD; this particular mutation has been chosen since it does not affect superoxide dismutase activity [23]. Several neuroblastoma cell lines have been established expressing constitutively either wild-type Cu,ZnSOD or the mutant enzyme. We have begun investigating alterations induced by FALS superoxide dismutases by determining modifications of mitochondria and cytosolic Ca^{2+} levels in these cell lines.

2. Materials and methods

2.1. Site-directed mutagenesis and cloning in expression vectors of Cu,Zn superoxide dismutase cDNAs

Plasmid pEMBLHSOD containing the hSODwt coding region was used to generate single stranded DNA molecules to be used in oligonucleotide-directed mutagenesis experiments as previously described [11].

cDNAs coding for wild-type and FALS SODs were then subcloned in expression vector pRc/CMV (Invitrogen) by PCR using oligonucleotides SOD1 (5'-GCAAGCTTATGGCTAAAGCTGTG-3') and SOD2 (5'-GCGGGCCCTTATTGGGCGATCCCAAT-3') as primers.

All clones were routinely verified by determination of their complete coding nucleotide sequences with Sequenase2 (USB).

2.2. Establishment and characterization of transfected cell lines

Human neuroblastoma SH-SY5Y cells were purchased from the European Collection of Cell Culture and grown in Dulbecco's MEM-F12 (Gibco) supplemented with 15% fetal calf serum, at 37°C in an atmosphere of 5% CO_2 in air.

Supercoiled plasmid DNA was purified by centrifugation on CsCl gradients essentially according to Sambrook et al. [12] and was introduced into cell cultures (10 μg for 5×10^6 cells) as a calcium-phosphate precipitate [13].

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Monoclonal cell lines were obtained by selection of cells in growth medium containing 500 μg/ml geneticin (G418 sulfate, Gibco) and manual isolation of clones; after selection the geneticin concentration was kept at 200 μg/ml. Extracts were obtained by disruption of cells through repeated freezing at −70°C and thawing, followed by centrifugation at 17000×*g* for 10 min. Supernatants were assayed for superoxide dismutase activity by the pyrogallol method at pH 8.2 [14] or by staining of non-denaturing discontinuous PAGE [15] according to Beauchamp and Fridovich [16]. Protein was determined according to Lowry et al. [17]. Evaluation of immunoreactive Cu,Zn-SOD was obtained by densitometric scanning of Western blots, performed on cell supernatants run on 12.5% SDS-PAGE using Immunblot kit from Bio-Rad and polyclonal antibodies against human Cu,ZnSOD raised in rabbits [18].

2.3. Determination of mitochondrial membrane depolarization

Changes in mitochondrial membrane potential were monitored in cells by determining the shift in fluorescence emission of the dye JC-1 (Molecular Probes, Eugene, OR). Depending upon the presence of an intact electrochemical gradient, JC-1 is able to form J-aggregates that are associated with a shift from green to orange fluorescence [19]. The electrochemical membrane potential was monitored by treating intact cells in suspension with JC-1 (10 μg/ml), progressively abolished by addition of valinomycin (0–5 μM) and determining FL2 fluorescence shift in a Facscan cytometer (Becton Dickinson) equipped with a standard filter set for green (530/30 nm) and orange (585/42 nm) emission [20].

2.4. Determination of intracellular calcium concentration

Control and transfected cell lines grown to about 70% confluence were incubated in growth media containing 1% FCS and 3 μM fluo-3AM (Molecular Probes) for 20 min at 25°C in the dark and then for a further 20 min at 37°C in the same conditions. After incubation with the fluorochrome, cultures were washed once in PBS and twice in HEPES saline; cells were then mechanically removed from the flask surface by repeated pipetting in HEPES saline, incubated for 10 min at 37°C and then directly tested for fluo-3 in a Facscan cytometer (Becton Dickinson) equipped with a standard filter set for green (530/30 nm) and orange (585/42 nm) emission. Maximum and minimum fluorescences were determined by addition of 5 μM ionomycin or 800 μM $MgCl_2$ before reading fluorescence intensity.

3. Results and discussion

Several SH-SY5Y neuroblastoma cell lines have been established, expressing either wild-type Cu,ZnSOD or G93A. SH-SY5Y cells constitutively express Bcl-2 [21], which is known to inhibit apoptosis without altering intracellular free Ca^{2+} [22]. This fact could be of help in establishing a model where over-

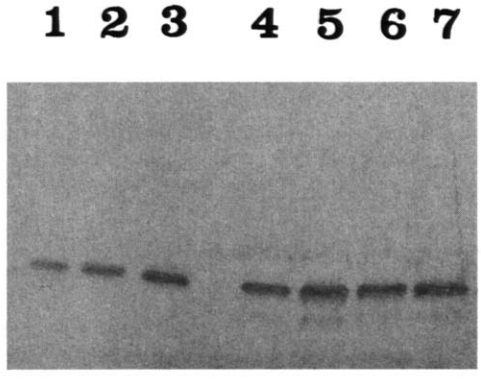

Fig. 1. Western blot analysis of 80 μg total protein from cell extracts from untransfected SH-SY5Y cells (control, lane 4), cells transfected with wild-type Cu,ZnSOD (lane 7) or with mutant Cu,ZnSOD (93 B, lane 5; 93 C, lane 6). Lanes 1–3 contained purified recombinant human Cu,ZnSOD (50, 100 and 200 μg, respectively).

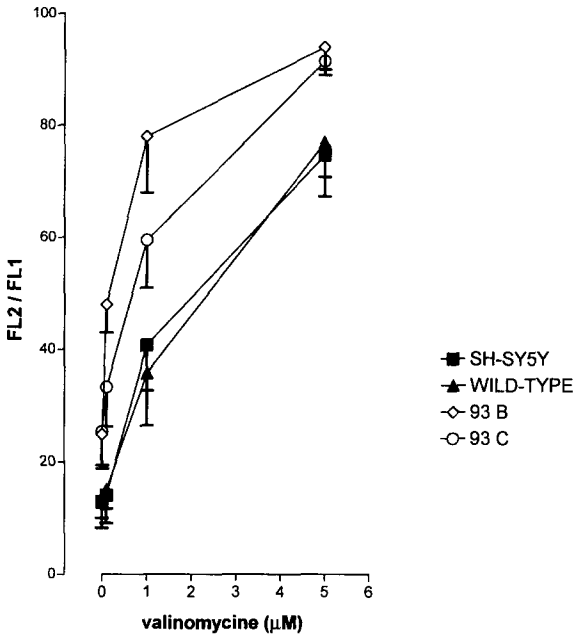

Fig. 2. Depolarization of mitochondria membranes by increasing concentrations of valinomycin, determined by JC-1 fluorescence shift. Mean values ± S.D. are reported; $n = 3$.

expression of the mutant enzyme might have led to rapid apoptosis and cell death. Many clones which had overcome the initial selection by geneticin did not survive in long-term culture: those lines might represent events in which overexpression of several copies of mutant SODs resulted in excessive damage and rapid cell death. We were able to propagate a total of six independent lines expressing G93A possibly at levels still compatible with survival of cells. However, even those lines quickly accumulated damage and after maintenance for 8–10 weeks in culture they were discarded and fresh aliquots were thawed. For further experiments we selected two independent monoclonal cell lines expressing mutant G93A (named 93B and 93C).

The level of Cu,ZnSOD immunoreactive protein has been determined by Western blot analysis. Fig. 1 shows that cells transfected with the plasmid encoding wild-type human Cu,ZnSOD possess a significantly higher content of immunoreactive enzyme as compared to untransfected SH-SY5Y cells. This is true also for lines 93B and 93C, where the mutants enzymes are expressed in a ratio close to 1:1 with the endogenous wild-type protein, a situation similar to that observed in heterozygous FALS patients. Total superoxide dismutase activity roughly matches the immunoreactive protein (Table 1), as expected on the basis of previous reports indicating that FALS SOD mutant G93A retains full SOD activity [23].

It has been demonstrated [24] that Cu,ZnSOD has a sec-

Table 1

Cell line	SOD activity (U/mg total protein)		SOD immunoreactive protein (arbitrary units)
SH-SY5Y	10.4 ± 1.2	$n = 6$	100
Wild-type	22.6 ± 1.2	$n = 4$	230
93 B	21.5 ± 3.3	$n = 4$	210
93 C	17.7 ± 1.9	$n = 4$	170

ondary, one-electron peroxidase activity having as substrates H_2O_2 and anionic molecules, including some neurotransmitters such as glutamate and taurine, which become free radicals by the peroxidase reaction. Some FALS mutants, including G93A, have higher rates than wild-type SOD1 in this reaction [23,25,26]. Mitochondria are a major cellular source of H_2O_2 [27], but they also represent a preferred target of this putative peroxidase reaction [28] due to the presence of sensitive lipids in their membranes. Peroxidative attack results in further production of H_2O_2 by the damaged respiratory chain. This vicious cycle ultimately results in mitochondrial swelling, uncoupling of oxidative phosphorylation, lipoperoxidation and dissipation of the Ca^{2+} gradient [29]. Indeed, transgenic mice carrying FALS mutation G37R present mitochondrial damage, selectively localized in neurons and uniformly absent in other non-neuronal cell types (i.e. glia) [10] despite significantly elevated free radical scavenging activity. When analyzed by staining with fluorochrome JC-1, our lines expressing mutant G93A showed a significant decrease in mitochondrial membrane potential compared with both the parental line SH-SY5Y and cells expressing wild-type SOD (Fig. 2). When depolarization of membranes was induced by increasing doses of valinomycin, lines 93B and 93C showed a higher sensitivity to this drug than both control cell lines (SH-SY5Y and WT).

The expected alteration in the long-term storage of calcium in damaged mitochondria of cells carrying mutated Cu,Zn-SODs was indeed indicated by the net increase of cytosolic Ca^{2+} concentration determined by fluo-3AM staining (Table 2). Again, this increase was found to be specific for cell lines expressing FALS-type mutants and was not related to total superoxide dismutase activity.

Both effects observed in our cell lines, damage of mitochondrial membrane and cytosolic Ca^{2+} increase, might offer a rationale for the highly selective vulnerability of the cortico-neuronal system in ALS. There is compelling evidence that free radicals together with increases in cytosolic Ca^{2+} play a major role in neuronal death, although neither the source of these radicals nor the direct connection between Ca^{2+} mobilization and radical production has been conclusively identified [30]. Ca^{2+} buffering potential in the cytosol seems to be crucial in determining the selective vulnerability to insults of certain populations of cells. Only trace amounts of immunoreactivity to the calcium buffering proteins calbindin-D28k and parvalbumin can be observed in human motoneurons [7] and this immunoreactivity is absent in motoneurons damaged early or severely in human ALS [31,32]. This process of neuronal injury may be self-sustaining. Elevation of cytosolic Ca^{2+} levels may compromise the structural integrity of mitochondria; it has been demonstrated that exposure to elevated Ca^{2+} induces production of free radicals in isolated mitochondria [30]. This could lead, in turn, to enhanced mitochondrial release of $OH^{\bullet}$ radicals and other reactive oxygen species.

Our data point to mitochondrial damage and Ca^{2+} levels as early factors in the pathogenesis of FALS. Whether mutations in Cu,ZnSOD induce this damage through generation of $OH^{\bullet}$ or other reactive species remains to be clarified and needs further investigation.

Table 2

Cell line	Cytosolic Ca^{2+} concentration (nM)	
SH-SY5Y	187 ± 7	$n = 8$
Wild-type	198 ± 13	$n = 5$
93 B	263 ± 22	$n = 6$
93 C	261 ± 23	$n = 4$

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