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Manganese Superoxide Dismutase Levels Are Elevated in a Proportion of Amyotrophic Lateral Sclerosis Patient Cell Lines

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The most frequent genetic causes of amyotrophic lateral sclerosis (ALS) determined so far are mutations occurring in the gene for copper/zinc superoxide dismutase (CuZnSOD). The mechanism may involve inappropriate formation of hyroxyl radicals, peroxynitrite or malfunctioning of the SOD protein. We hypothesized that undiscovered genetic causes of sporadically occurring amyotrophic lateral sclerosis might be found in the mechanisms that create and destroy oxygen free radicals within the cell. After determining that there were no CuZnSOD mutations present, we measured superoxide production from mitochondria and manganese superoxide dismutase (MnSOD), glutathione peroxidase, NFκB, Bcl-2 and Bax by immunoblot. Of the ten sporadic patients we tested we found three patients with significantly increased concentrations of MnSOD. These patients also had lower levels of superoxide production from mitochondria and decreased expression of Bcl-2. No mutations were found in the cDNA sequence of either MnSOD in any of the sporadic patients. A patient with a CuZnSOD mutation (G82R) used as a positive control showed none of these abnormalities. The patients displaying the MnSOD aberrations showed no specific distinguishing features. This result suggests that the cause of ALS in a subgroup of ALS patients (30%) is genetic in origin and can be identified by these markers. The alteration in MnSOD and Bcl-2 are likely epiphenomena resulting from the primary genetic defect. It suggests also that the oxygen free radicals are part of the cause in this subgroup and that dysregulation of MnSOD or increased endogenous superoxide production might be responsible. © 2000 Academic Press

At least three phenotypically defined forms of familial ALS exist, all inherited in an autosomal dominant fashion (1). Typically there is rapidly progressive loss of motor function, with mainly the anterior horn cells and pyramidal tracts affected. Alternatively there is identical clinical presentation and progression but with the pathological changes including changes in posterior columns and spinocerebellar tracts (2–4). Variations do seem to occur in the age of onset and duration of the disease and one or both of these parameters is often consistent within an affected family over a number of generations (5–9). While 10% of ALS is familial, sporadic ALS (SALS) accounts for the other 90%, but this does not mean that the disease does not have a genetic basis. The discovery of mutations in the CuZnSOD gene in the familial group showed that ALS could be a disorder of oxygen free radical processing (10, 11) and that these mutations could operate either in a dominant or recessive manner (12). Enzymes have evolved with the task of detoxifying the primary oxygen free radical, superoxide, collectively being named the superoxide dismutases. There are three of them in mammalian systems: a cytosolic CuZn superoxide dismutase (SOD1), an intramitochondrial manganese superoxide dismutase (MnSOD or SOD2) and an extracellular CuZn superoxide dismutase (SOD3) (13). MnSOD, located in a separate compartment from CuZnSOD, is necessary because the respiratory chain assembly of mitochondria which carries out the process of electron transport produces superoxide in substantial amounts as an obligatory by-product of its activity.

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Indeed it has been estimated that 1–2% of all electrons passing down the respiratory chain are diverted to produce superoxide (14). Other enzyme systems are present to remove the hydrogen peroxide, the major task going to mitochondrial and cytosolic versions of the enzyme glutathione peroxidase (15).

In this study we took a group of sporadic ALS patients, and using skin fibroblasts grown in identical conditions, investigated the enzymes and proteins involved in the defense against damage from oxygen free radicals.

MATERIALS AND METHODS

Patients. Ten adult patients with an established diagnosis of SALS were recruited to this study. Age of onset varied from 38 to 69 and they were given an overall classification of limb or bulbar based on the predominant symptoms, all with ALS as defined by "El Escorial" criteria. One patient with a proven CuZnSOD mutation (G82R) was included as a positive control (16). The identity and phenotypic diagnoses were known only to the referring neurologist while the study was being conducted and were unmasked after all the investigations were complete and tabulated. Control patient material in the form of cultured skin fibroblasts was derived from the cell repository at the Hospital for Sick Children, Toronto and at Montréal Children's Hospital.

Cell cultures. Human fibroblasts were derived from skin biopsies from the patients described above with informed consent. All fibroblast cell lines were cultured in Eagles alpha-minimal essential medium supplemented with 10% fetal calf serum, 10.5 mM glucose, and 1 mM uridine. Care was taken to use the cells before passage number 7 for our studies and observations were repeated at least twice on all cell lines.

Immunoblotting. The patients were compared using Western blot analysis for Bcl-2, MnSOD and Bax, glutathione peroxidase and NFκB in either mitochondrial or cytosolic fractions as indicated.

Mitochondrial preparation. Mitochondria were isolated from cultured skin fibroblasts as previously described using a mitochondrial isolation buffer [0.34 M sucrose, 100 mM KCl, 10 mM Tris–Cl, 1 mM EDTA at pH 7.4] (16). Protein concentration was determined by the method of Lowry *et al.* (16). Mitochondria were frozen in liquid nitrogen after isolation and stored at −70°C prior to use (17).

Superoxide assay system. Superoxide production was estimated by luminometry using lucigenin as the reporter molecule (18). The NADH dependent superoxide production rate (nmol/min/mg) with NADH as substrate was estimated using a method similar to that used by Pitkänen *et al.* (19).

Antibodies. The rabbit anti-Bcl-2 and anti-Bax antibodies were from Calbiochem (San Diego, CA). Rabbit anti-MnSOD against a peptide encoding the last 14 C-terminal amino acids (209–222) of the protein and anti-rabbit complex I (CI) 49 K encoding the residues near the C-terminus residues 416–430 were raised by Research Genetics Inc. (Huntsville, AL). Glutathione peroxidase was purchased from Sigma Chemical Co. (St. Louis, MO) and used to raise antibody in rabbits. NFκB antibody (p65 and p50) were purchased from Santa Cruz (Santa Cruz, CA).

Western blotting. Mitochondrial proteins (100 μg for fibroblasts and 50 μg for muscle) were separated on 18% SDS–PAGE gel and transferred to a 0.2 mm nitrocellulose membrane. The membranes were blocked with 5% non-fat dry milk in blotto [10 mM Tris–Cl pH 7.4, 150 mM NaCl, 0.2% Tween 20] for 1 h at room temperature and washed with phosphate buffered saline (PBS). The blots were incubated with the primary antibodies (1:1000 dilution) in 3% BSA/PBS for 16 h at room temperature. These were then washed and incubated with a secondary anti-rabbit IgG alkaline phosphatase conjugate antibody (Bio-Rad Laboratories, Hercules, CA) in 3% BSA/PBS for 2 h at room temperature. Membranes were washed with PBS, and developed using BCIP/NBT detection system (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's protocol. The intensity of visualized bands was estimated using NIH Image version 1.57 (National Institute of Health, Bethesda, MD).

RESULTS

At the outset of this study 14 patients with ALS were selected for analysis. With informed consent these patients gave skin biopsies for analysis for evidence of abnormalities involving oxygen free radicals. Each skin fibroblast culture was established by a standard protocol, but only ten of these biopsies yielded sufficient cells to complete the study. From each patient culture exactly the same method was used to make isolated mitochondria. In the making of mitochondria a post 12,000 g supernatant was kept as a cytosolic fraction for estimation of NFκB and cytosolic glutathione peroxidase. One cell line from a familial patient with a mutation in CuZnSOD (G82R) was used as a positive control for SOD1-ALS, and three control cell lines were used initially to test if there was any natural variation in parameters from cell line to cell line.

MnSOD (SOD2) measured by immunoblot was found to be markedly elevated in the mitochondria of three patients and partially elevated in one other. MnSOD levels were remarkably stable in mitochondria from control cell lines, there being little variation between cell lines. This echoes studies done previously with control cell lines compared to those from patients with respiratory chain defects. Three control cell strains used in that study had uniformly low levels of MnSOD, as was observed here (19). The three control cell lines used in this present study were of different origin, giving a total of six cell lines surveyed. A further four control cell lines were then examined and these also had no elevation of MnSOD. We therefore concluded that the elevations in three out of ten ALS cell lines were significant. Dual blots performed with anti-MnSOD and anti-49 kDa complex I subunit on three patients and one control show that the MnSOD varies relative to the stable complex I 49 kDa subunit antibody detectable band (Fig. 1).

Superoxide production measured in mitochondrial membranes prepared from the skin fibroblasts was evaluated by lucigenin luminescence assay (18). In the mitochondria prepared from the skin fibroblasts of patients with elevated MnSOD the superoxide production was half that in the control cell lines. In the other patients it was no different from the controls. Glutathione peroxidase was measured by immunoblot in both cytosolic and mitochondrial fractions from both patient and controls and was found to be remarkably constant in titre in both compartments. NFκB was also

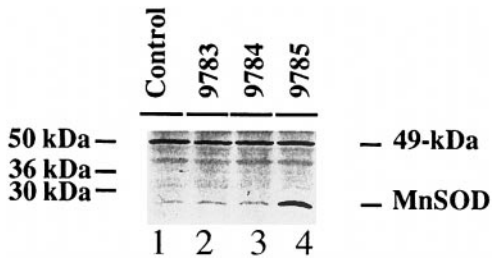

FIG. 1. Immunoblot of ALS skin fibroblast mitochondria. Immunoblotting procedures were carried out as described under Materials and Methods using antibodies for MnSOD and the 49 kDa subunit of complex I. Lane 1, control skin fibroblasts; Lane 2, ALS patient 9783; Lane 3, ALS patient 9784; Lane 4, ALS patient 9785.

unchanged as was the pro-apoptotic protein Bax measured in the mitochondrial fraction. The anti-apoptotic Bcl-2 protein, again measured in mitochondria, however, showed a decrease in three out of the four cell lines of ALS patients that had increased MnSOD (Fig. 2). In other ALS cell lines, including the CuZnSOD mutation-bearing cell line and controls, expression of Bcl-2 and Bax was constant. A summary of these results is shown in Table 1.

DISCUSSION

The increased MnSOD levels and decreased superoxide production from these three out of ten ALS derived cell lines are significant findings. Knowing that reactive oxygen species (ROS) are instrumental in cell damage, these findings strongly suggest that oxygen free radicals may play a significant role in the disease pathogenesis mechanism operating in these individuals. Because of concerns over the possibility of a link between MnSOD expression and variables connected with the ageing process, care had to be taken with these cell lines to make sure that they were of equivalent passage number and not senescent. In addition we carefully followed control cell lines throughout their lifetime. We did not observe any increase in MnSOD expression with passage number or with senescence that might be an explanation for the observed increases in three of the ALS subjects. A total of ten non-disease control cell lines have been examined in this study and in previous studies by us and no such increase in MnSOD was ever observed in any of them. We have examined the MnSOD levels in the fibroblast mitochondria of >100 patients with diseases other than ALS and find significant alterations in only those patients with complex I deficiency (19). The finding of decreased superoxide production in mitochondrial membranes from affected cell lines was not unexpected. In previous studies we found that mitochondria with increased levels of MnSOD showed a decrease of measurable superoxide when primed with NADH because the superoxide was preferentially removed by the superoxide dismutase (18, 19). The same effect could be produced by adding purified CuZnSOD to the mitochondrial preparation before eliciting the superoxide response (18).

In our previous studies of elevated superoxide dismutase in mitochondria, the elevations were present because of defects in NADH-ubiquinone oxidoreductase, which led to changes in redox state and increased free radical production. In these cells there was no evidence of any respiratory chain defect. Measurement of lactate/pyruvate ratio in the cells and NADH-cytochrome c reductase in the mitochondria of the cells with elevated MnSOD showed both variables were not different from those measured in controls (results not shown). Therefore the origin of the elevation of MnSOD in these cells does not seem to lie in a change in redox state or in an overtly defective respiratory chain. It is possible therefore that the defect might lie instead in the mechanisms that control induction and repression of MnSOD and that the observed elevation is compensatory.

We found previously that there were two reciprocal mechanisms at work in mitochondria at risk from damage by oxygen free radicals due to defects in the mitochondrial respiratory chain. In some cells there was an induction of Bcl-2 so that the Bcl-2/Bax ratio was significantly increased. In other cells there was an induction of MnSOD, which seemed to be a response to a dual change in both redox state and in superoxide production (20). In the cells from the current study there was a lack of the protective Bcl-2 in cells that had increased their MnSOD, again highlighting this reciprocal behaviour, seen at work before in cultured skin fibroblasts. Bcl-2 has been shown to be protective against free radical induced apoptosis in both yeast and mammalian cells (21). In a mouse model of ALS (G93A CuZnSOD), which mimics many of the symptoms of ALS, crossed with a mouse overexpressing a Bcl-2 transgene, the offspring with both fully expressed transgenes were protected from neuronal damage (22). In skin fibroblasts it is not exactly clear how

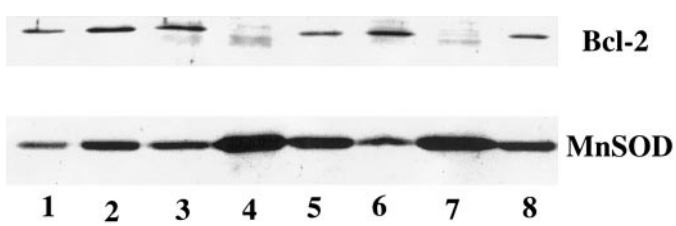

FIG. 2. Immunoblot of ALS skin fibroblast mitochondria for bcl-2. Immunoblotting procedures were carried out as described under Materials and Methods using antibodies for bcl-2 and MnSOD in separate blots with the same samples. The results are combined in this figure. Lane 1, ALS patient 12636; Lane 2, ALS patient 12620; Lane 3, ALS patient 12618; Lane 4, ALS patient 12542; Lane 5, ALS patient 12510; Lane 6, ALS patient 12494; Lane 7, ALS patient 12493; Lane 8, control 9152.

TABLE 1

Summary of the Findings for Ten Sporadic ALS Patients, One SOD1 (G82R) Patient, and Controls for Immunoblots, Superoxide Measurement, and Clinical Status

Cell line	MnSOD	49K Complex I	Bcl-2	Bax	Superoxide	GPX	NFκB	Phenotype	Age (onset)
9783 (CuZnSOD)	+	++	+	+	++	ND	+	Familial	29
9784	+	++	−	+	++	ND	+	bulbar	48
9785	+++	++	+	+	+	++	+	limb	53
12493	+++	++	−	+	+	++	+	limb	35
12494	+	++	+	+	++	++	+	limb	41
12509	++	++	−	+	+	++	+	bulbar	54
12510	+	++	+	+	++	++	+	limb	63
12542	+++	++	−	+	+	++	+	limb	52
12618	+	++	+	+	++	++	+	bulbar	65
12620	+	++	+	+	++	++	+	bulbar	46
12636H	+	++	+	+	++	++	+	limb	67
Controls	+	++	+	+	++	++	+		

Note. Determinations were carried out as described under Materials and Methods. Patients are scored as increases or decreases relative to the situation in controls.

this reciprocal system works. While mechanisms for MnSOD induction are known to include NFκB dependent promoter driven transcription (23), we could find no evidence of changes in NFκB titre in nucleus or cytoplasm in any of the high titre MnSOD ALS lines (24). Protein kinase C (PKC) dependent mechanisms also exist which influence MnSOD gene transcription as do PKC mechanisms for Bcl-2 phosphorylation, so it is here possibly that there is some connection (24–26).

Since it is unlikely that ALS patients are actually suffering from mitochondrial respiratory chain defects, it is possible that the defect leading to increased MnSOD and decreased Bcl-2 perhaps is associated with some maladjustment in one of these signalling mechanisms. The net result of having too much MnSOD over a prolonged period could be the overproduction of hydroxyl radicals formed from the excess hydrogen peroxide generated. Increased production of hydrogen peroxide in the cytosolic compartment or perhaps production of OH radical by back reaction of hydrogen peroxide through CuZnSOD (27) has been theorized as being responsible for making CuZnSOD overexpressing mice more susceptible to radiation, infection and other stresses (28, 29). The overexpression of mitochondrial MnSOD in fibroblasts inhibited growth while overexpression in rat glioma cells made those cells more sensitive to damage by radiation and carcinogens (30, 31). Thus there is a precedent for overactivity of superoxide dismutases being deleterious but as an alternative there could be another aberrant source of superoxide production which we have not investigated that lies at the base of this problem. Overexpression of MnSOD in brain stem motor neurons from patients with ALS has been noted in patients in a previous study (32). Overall, the net result of increased MnSOD in three of ten ALS patient derived cell lines strongly suggests a further genetic mechanism involving oxygen free radicals is responsible for ALS. It also suggests that this association should be investigated in a larger cohort of patients, and mechanisms governing MnSOD expression be investigated in association with ALS.

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