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Down-regulation of copper/zinc superoxide dismutase causes apoptotic death in PC12 neuronal cells

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ABSTRACT **The discovery of missense mutations leading to reduced enzymatic activity in the copper/zinc superoxide dismutase (SOD1) in human familial amyotrophic lateral sclerosis has heightened interest in the role of free radicals in neurodegenerations but left the mechanisms by which they may cause neuronal death unexplained. We have approached this problem by specifically inhibiting the synthesis of SOD1 in cultured PC12 cells with antisense oligonucleotides. Cell survival in both untreated and nerve growth factor (NGF)-treated PC12 cells was inhibited by down-regulation of SOD1, with NGF-treated cells dying at lower levels of inhibition than untreated cells. Dying cells showed DNA degradation characteristic of apoptosis and could be rescued by the antioxidant vitamin E, with a combination of vitamin E and NGF being most efficacious. These results suggest that the induction of cell death by inhibition of SOD1 is due to free radical induction of apoptosis and that growth factor therapy for free-radical-mediated disease may require antioxidants in order to be effective.**

Among the many ways in which cells may be damaged is through the action of free radicals. Free radicals may function as intermediate steps in destructive pathways triggered by other agents, or directly as a result of oxidative stress. In the nervous system, free radicals have been invoked in the pathogenesis of Parkinson disease (1) and more recently in a familial form of amyotrophic lateral sclerosis (FALS) (2, 3). In this latter condition, missense mutations in the gene encoding the copper/zinc superoxide dismutase (SOD1) (2) result in structural defects and decreased SOD1 enzymatic activity (3). SOD1 is just one player in the mechanisms which have evolved to allow cells to deal with free radicals (4). Others include the Mn^{2+} SOD (5), scavenger molecules such as α-tocopherol (vitamin E), the glutathione system, and catalase (4). In these pathways, the SODs catalyze the conversion of the superoxide radical to hydrogen peroxide while glutathione peroxidase and catalase clear peroxide, preventing the production of hydroxyl radicals, the initiators of lipid peroxidation (1). Under physiological conditions there is an equilibrium between oxidant and antioxidant mechanisms. Changes in this balance in favor of free-radical formation would result in oxidative stress and favor neuronal damage (6). An understanding of the mechanism by which decreased SOD1 activity could lead to neuronal loss in ALS is critical to unraveling the pathogenesis of the disease and devising rational therapeutic approaches.

In the experiments presented here we have used the ability to specifically down-regulate SOD1 in cells with antisense oligonucleotides to study the effects of decreased SOD1 activity on cultured neuronal cell viability.

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MATERIALS AND METHODS

Cell Culture. PC12 rat pheochromocytoma cells were grown as described (7) on rat-tail collagen-coated dishes in RPMI 1640 medium containing 5% fetal bovine serum and 10% heat-inactivated horse serum, with and without mouse submaxillary-gland nerve growth factor (NGF, 50 ng/ml). For incubation with oligonucleotides, cells were washed three times with serum-free RPMI 1640 and then plated on fresh collagen-coated dishes in RPMI 1640 supplemented with insulin (3 μM) with and without NGF (50 ng/ml) (8).

Oligonucleotides. Antisense and sense oligonucleotides were synthesized by Operon Technologies (Alameda, CA). Two different SOD antisense oligonucleotides [rat SOD1 sequence (9)], each 21 bases long, were synthesized: ASOD1α (bases 54–74, initiation codon at 59) and ASOD1β (bases 497–517). Also synthesized were the sense construct of ASOD1α and a nonsense construct (same base composition as ASOD1α, but scrambled).

Immunofluorescence. PC12 cells, washed as above, were plated on Lab-Tek chamber slides and grown overnight in the presence and absence of antisense oligonucleotides (at 2 μM and 20 μM). Cells were fixed in ice-cold methanol and stained with anti-SOD1 (Sigma), a monoclonal antibody prepared against human SOD1, which crossreacts with rat SOD1. The primary antibody was visualized with fluorescein isothiocyanate-conjugated goat anti-mouse (10).

Cell Viability. Cells were grown in 24-well dishes and lysed in 200 μl of a solution which lyses the cell membrane but leaves the nuclei intact (11). The nuclei were counted in a hemocytometer. Viability was also assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (12). Metabolic activity was measured by 2-[^{3}H]deoxyglucose uptake (13) studies in which equal numbers of cells were plated in 35-mm plates, incubated with 2-[^{3}H]deoxyglucose for 2 hr and extracted for scintillation counting.

SOD Specific Activity. Cells were extracted with 0.5% Nonidet P-40 and protein was measured by the Bradford method (14). SOD1 levels were determined with a modification of the xanthine/xanthine oxidase system (15), measuring the reduction of nitroblue tetrazolium at 560 nm in the presence and absence of KCN (16). Briefly, cell extracts or SOD (Sigma) were incubated in 50 mM sodium carbonate buffer (pH 10.2) containing 0.1 mM EDTA, 0.1 mM xanthine, 1 mM KCN, 25 μM nitroblue tetrazolium, and 2.2 nM xanthine oxidase in a volume of 1 ml. Reduction of nitroblue tetrazolium was measured at 560 nm. SOD1 activity was determined from SOD standard curve and is reported as the KCN-sensitive activity.

Extraction of DNA and Southern Blot Analysis. Cells were extracted and analyzed as described (17). PC12 cells (3–10 $\times$ 10^6 per condition, with equal amounts of cells per condition in each experiment) were washed and plated in uncoated

Abbreviations: ALS, amyotrophic lateral sclerosis; FALS, familial ALS; NGF, nerve growth factor; SOD, superoxide dismutase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

60-mm plastic culture dishes in RPMI 1640 medium with and without additive. After incubation for the indicated times, cells were triturated off the dishes and centrifuged at 800 × g for 5 min. Soluble DNA was extracted by the method of Hockenberry *et al.* (18) and resuspended in TE buffer (10 mM Tris/1 mM EDTA, pH 7.4). Samples were incubated with DNase-free RNase (50 μg/ml). All the soluble DNA recovered per condition was subjected to electrophoresis in a 1.4% agarose gel and blotted onto GeneScreen*Plus* membrane (DuPont/NEN). Blots were probed with total genomic PC12 cell DNA digested with *Sau*3A1. ^{32}P-labeled probe was prepared by the random priming reaction (19), and hybridization and washings were performed according to the manufacturer's protocol (DuPont/NEN).

RESULTS

Antisense Oligonucleotides to SOD1 Decrease Immunohistochemically Detected SOD1. Several antisense oligonucleotides based on the sequence of rat SOD1 were constructed and their ability to decrease immunohistochemically detectable SOD1 was assessed with a commercially available monoclonal antibody (Sigma). This antibody gave strong cytoplasmic staining in PC12 cells. Two of the antisense oligonucleotides, ASOD1α (bases 54–74, initiation codon at 59) and ASOD1β (bases 497–517), decreased SOD1 immunofluorescence in PC12 cells within 24 hr of treatment. Since ASOD1α was effective at lower concentrations than ASOD1β, all subsequent studies were done with ASOD1α (ASOD1). Neither the sense construct of ASOD1α nor a nonsense construct (same base composition as ASOD1α, scrambled) affected anti-SOD1 immunostaining.

Inhibition of SOD1 in PC12 Cells Results in Cell Death. To evaluate the effect of decreasing SOD1 on cell viability, PC12 cells were transferred to serum-free RPMI 1640 medium supplemented with insulin (8). Serum-free conditions were necessary to avoid rapid degradation of the oligonucleotides by serum endonucleases. In the absence of either insulin or NGF, PC12 cells fail to survive in serum-free conditions (20, 21). Exposure of the cells to ASOD1 for 24 hr caused death in both naive and NGF-pretreated (10–14 days with NGF) PC12 cells. Loss of cell viability was determined by counting nuclei after cell lysis (Fig. 1). Nuclear counting yields a more accurate estimate of cell viability than methods such as trypan blue exclusion because PC12 cells grow in clumps, rendering counting of individual cells difficult. Confirmation of cell viability was obtained by MTT uptake under the same conditions presented in Fig. 1. NGF-treated cells were more susceptible, with 50% loss seen at an ASOD1 concentration of 1 μM compared with 4 μM for the untreated cells (Fig. 1). This difference between naive and NGF-treated cells raised the issue of relative metabolic activity of the various cells. Therefore, the metabolic activity of naive and NGF-treated cells was assessed with 2-[^{3}H]deoxyglucose uptake. Uptake of 2-deoxgylucose was the same in triplicate cultures of naive and 10-day-NGF-treated cells. The sense and nonsense constructs did not affect SOD1 levels or cell survival, even at concentrations 10 times those used for the antisense constructs.

SOD1 activity in PC12 cultures fell rapidly after addition of ASOD1, reaching a level 50% of control in 5 hr, a time at which there was little or no cell death (Fig. 2). In spite of the lower dose of ASOD required to kill 50% of the NGF-treated cells, the kinetics of SOD1 inhibition were similar. In control, untreated PC12 cells virtually all the SOD activity was KCN-sensitive, whereas in control NGF-pretreated cells 70% was KCN-sensitive, indicating induction of additional, non-SOD1, antioxidant activity by NGF. The down-regulation of SOD1 in untreated cells by ASOD1 led to an increase in non-SOD1 (KCN-insensitive) activity, to the levels found in the NGF-pretreated control cells (Table 1). However, NGF-pretreated cells showed no further increase on ASOD1 treatment, suggesting that their antioxidant capacity was maximally induced by NGF. SOD1 activity was the same in PC12 cells whether they were grown serum-free, with or without growth factors (insulin or NGF), or in the presence of serum.

Apoptotic Internucleosomal Cleavage Occurs in ASOD1-Induced Cell Death. PC12 cells have been shown to die both by apoptosis and by necrosis, depending on the mode by which death is induced (14). When PC12 cells are cultured in serum-free RPMI 1640 medium, they exhibit DNA fragmentation and the morphologic changes characteristic of apoptosis (19). We carried out Southern blot analysis of soluble DNA from PC12 cells treated with ASOD1, using radioactive probes derived from PC12 total genomic DNA. A clear pattern of internucleosomal DNA cleavage (discrete frag-

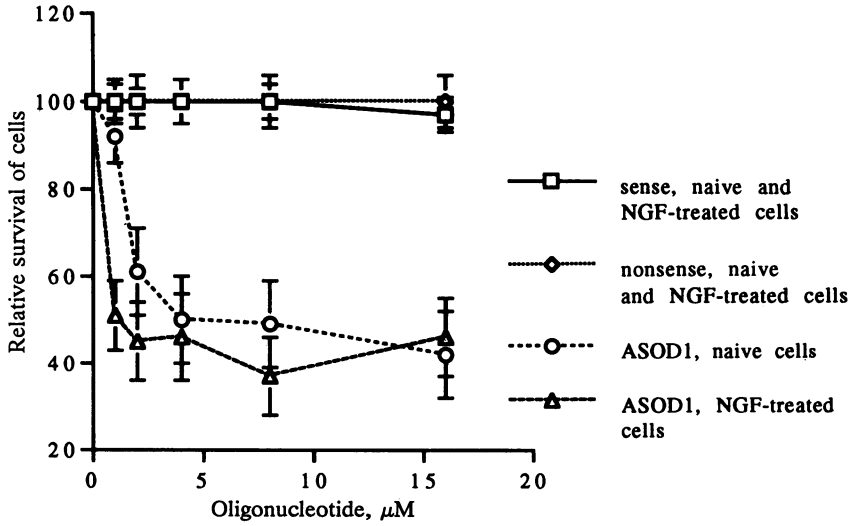

FIG. 1. Relative PC12 cell survival after treatment with oligonucleotides. Naive or NGF-pretreated PC12 cells (treated for 10–14 days with NGF at 50 ng/ml) were washed and plated in serum-free RPMI 1640 supplemented with 3 μM insulin and then incubated with indicated concentrations of the various oligonucleotides (ASOD1, sense construct of ASOD1, and a nonsense scrambled construct) for 24 hr. Cells were lysed and nuclei were counted; the number of surviving cells is expressed relative to the number present without oligonucleotide (designated as 100). Values represent means ± SEM (n = 5). Two-way analysis of variance was used to analyze data at each concentration; P values ranged from 0.0001 (for 1 μM) to 0.006 (for 8 μM); the P value for 16 μM was not significant.

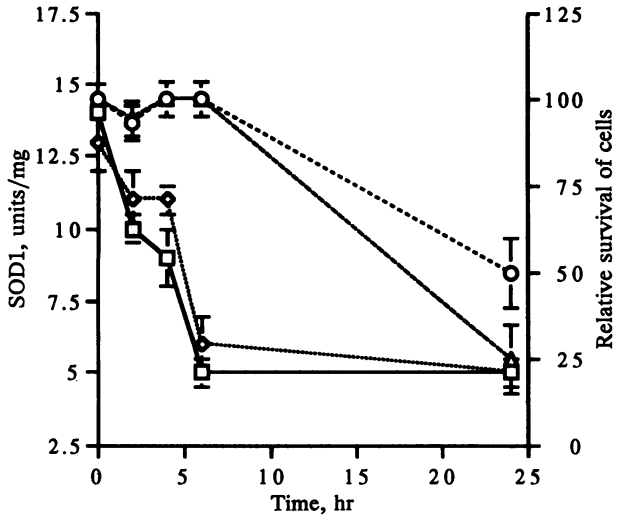

FIG. 2. SOD1 specific activity over 24 hr with ASOD1. Naive (□, ○) and NGF-pretreated (◇, △) PC12 cells were incubated with ASOD1 (8 μM). Cells were extracted with 0.5% Nonidet P-40 and protein was measured by the Bradford method. SOD1 levels were determined with the xanthine/xanthine oxidase system, with measurement of the reduction of nitroblue tetrazolium at 560 nm in the presence and absence of KCN, at the indicated times. SOD1 activity (□, ◇) was determined from a SOD standard curve and is reported as the KCN-sensitive activity. The relative number of surviving cells (○, △) was determined as described for Fig. 1, at the indicated times. Values represent means ± SEM (n = 3).

ments differing in size by multiples of ~180 bp) was evident after 7 hr of treatment with ASOD1 (Fig. 3). There was no evidence of cleavage at 3 hr, at which point cell loss was not apparent, or at 24 hr, when most cells were dead. Aurintricarboxylic acid, a general inhibitor of nucleases, suppresses apoptosis and promotes long-term survival of PC12 cells in serum-free cultures (19). In our system, aurintricarboxylic acid (100 μM) suppressed the endonuclease activity produced by treatment with ASOD1. Cycloheximide at 10 μg/ml, a level that blocks protein synthesis by 90% (22), did not prevent ASOD1-induced internucleosomal DNA cleavage.

Vitamin E Suppresses ASOD1-Induced Cell Death. The role of free radicals in cell death in these cells is supported by their rescue by the antioxidant vitamin E. Pretreatment of PC12 cells with vitamin E suppressed ASOD1-induced cell death in both untreated and NGF-pretreated PC12 cells (Fig. 4). Rescue of cells by vitamin E began at lower concentrations for naive cells (<1 μg/ml) than for NGF-pretreated cells (10 μg/ml). However, at higher concentrations the combination of vitamin E and NGF resulted in greater rescue than vitamin E alone. The down-regulation of SOD1 by ASOD1 was unaffected by vitamin E (Table 2), ruling out a nonspecific interaction of vitamin E with the oligonucleotide.

DISCUSSION

Our results show that a decrease of SOD1 in PC12 cells to <40% of constitutive levels results in rapid cell death by

Table 1. Distribution of SOD activity before and after down-regulation of SOD1

	SOD1, units/mg	SOD2, units/mg
Naive	14 ± 1	0 ± 0.5
Naive + ASOD	5 ± 0.5	4.2 ± 0.5
NGF	13 ± 1	3.9 ± 0.5
NGF + ASOD	5 ± 0.5	3.9 ± 0.6

SOD1 activity is the KCN-sensitive fraction of total SOD activity, whereas SOD2 activity is the KCN-insensitive fraction, determined as described in *Materials and Methods*. Values represent means ± SEM (n = 3).

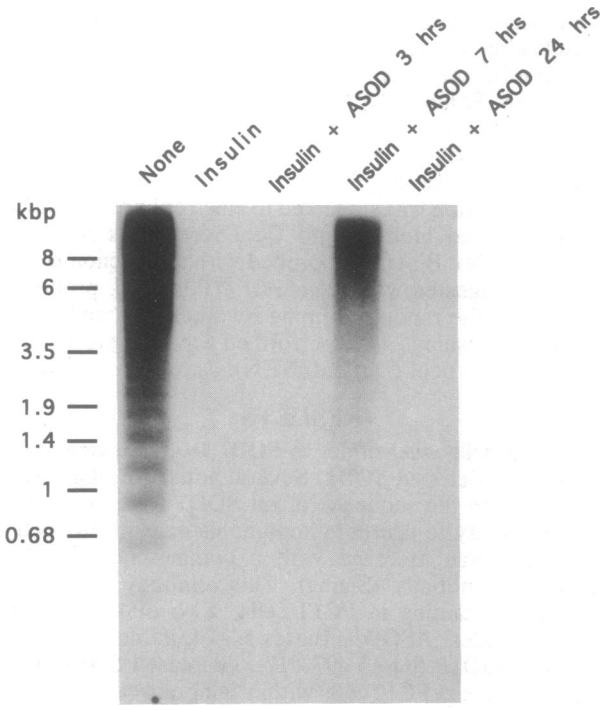

FIG. 3. Southern blot of soluble PC12 cell DNA. PC12 cells were washed with and cultured in serum-free RPMI 1640 in the presence of no additives (None) for 24 hr, 3 μM insulin for 24 hr, or 3 μM insulin with 8 μM ASOD1 for the indicated times. Soluble DNA was extracted from the cells and analyzed as described in *Materials and Methods*.

apoptosis. That the death is inhibited by vitamin E suggests that this mechanism is free-radical-mediated and not due to some other effect of down-regulating SOD1.

While free radicals may cause cellular damage in a variety of ways, in the experiments reported here they led to internucleosomal DNA cleavage and apoptotic cell death. Apoptosis may be due to the attack of free radicals, specifically

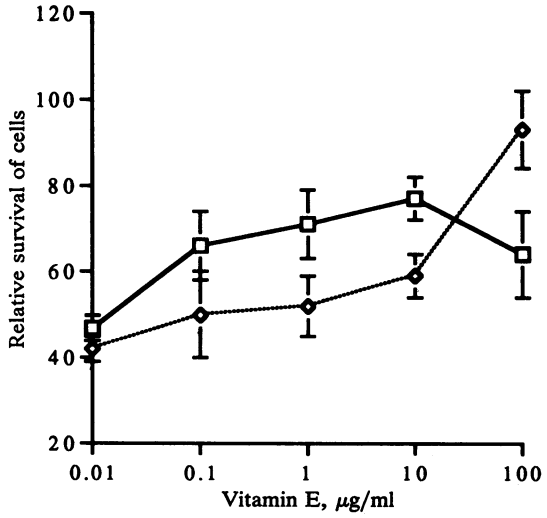

FIG. 4. Vitamin E suppression of cell death in the presence of ASOD1. Naive (□) or NGF-pretreated (◇) PC12 cells were incubated for 4 hr with the indicated concentrations of vitamin E and then for an additional 20 hr with ASOD1 (8 μM). Cells were lysed and nuclei were counted; the number of surviving cells is expressed relative to the number present without oligonucleotide (designated as 100). Values represent means ± SEM (n = 5). Two-way analysis of variance at each concentration gave P values < 0.002 for 0.1–100 μg/ml.

Table 2. Effect of vitamin E on down-regulation of SOD1

Cells	SOD1, units/mg	
	ASOD	Vitamin E + ASOD
Naive	5 ± 0.5	4.7 ± 0.5
NGF-treated	5 ± 0.5	4.5 ± 0.5

Naive and NGF-pretreated PC12 cells were incubated for 20 hr with ASOD1 (8 μM) or for 4 hr with vitamin E (10 μg/ml and 100 μg/ml, respectively) and then for an additional 20 hr with ASOD1 (8 μM). SOD1 specific activity was determined as described in *Materials and Methods*. Values represent means ± SEM (n = 3).

the hydroxyl radical, on the DNA, increasing its susceptibility to endonuclease cleavage. A related effect is seen in *Escherichia coli* where exposure to low doses of peroxide leads to killing caused by DNA damage while high-dose exposure leads to killing via an undetermined mechanism (6). Although data on the effects of decreasing neuronal SOD1 are lacking, *Drosophila* with a null mutation in the copper/zinc SOD are infertile and have a reduced lifespan (23). *E. coli* cells lacking SOD exhibit enhanced mutation rates during aerobic growth, supporting the concept of direct DNA damage as a result of the absence of SOD (24). Recent work has demonstrated that *bcl-2*, a protooncogene which prevents apoptotic death in several systems, protects against peroxide-induced death (25).

In view of widespread interest in the use of neurotrophic factors for the treatment of neurodegenerative diseases, it was surprising to find that NGF-treated PC12 cells were more sensitive to the down-regulation of SOD1 than untreated cells. Half of the NGF-treated cells died after treatment with 1 μM ASOD, whereas naive PC12 cells required 4 μM ASOD (Fig. 1). In each case, the down-regulation of SOD1 showed the same kinetics (Fig. 2) and the metabolic activities, as measured by uptake of 2-deoxyglucose, were the same. The NGF-treated cells also required larger amounts of vitamin E to rescue them than did untreated cells (Fig. 4). However, the combination of NGF and higher doses of vitamin E was more efficacious than high-dose vitamin E alone. These paradoxical results may be explained by consideration of the levels of oxidant stress (or reserve) in the NGF-treated and untreated cells prior to downregulation of SOD1. In NGF-treated PC12 cells 30% of the antioxidant activity is non-SOD1, while in naive PC12 cells virtually all of the activity is SOD1. Upon treatment with ASOD, naive PC12 cells show an induction of non-SOD1 antioxidants equal to 30% of pretreatment total antioxidant levels, whereas NGF-treated cells show no change in non-SOD1 levels (Table 1). This suggests that NGF-treated cells are already under oxidative stress and have totally mobilized their antioxidant reserves. Lacking these reserves, the NGF-treated cells succumb at a lower dose of ASOD1. Our studies suggest that when SOD1 is reduced, the superoxides produced are toxic, an effect which is reversed by the supplementation of antioxidant activities by the scavenger vitamin E. A similar effect is possible in the therapeutic situation when an excess of exogenous neurotrophic factor is delivered. The increased trophic drive could result in the generation of more free radicals than the cell has the capacity to inactivate. Potentially, this problem could be alleviated by concurrent antioxidant therapy.

It appears that the vulnerability of cells to conditions or agents which affect the generation and inactivation of free radicals depends on the extent of their ambient oxidative load and their antioxidant reserve. This balance could be the basis for selective vulnerability of certain neurons and the resistance of others in conditions such as FALS and Parkinson disease. Diseases of late onset and slow progression may reflect the gradual deterioration caused by chronic small imbalances in these factors. Further studies on primary neurons, especially motor neurons, will be necessary to determine whether the free-radical induction of apoptosis seen in our model system provides an appropriate model for FALS and other degenerative neuronopathies.

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