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Mutant Superoxide Dismutase-1-Linked Familial Amyotrophic Lateral Sclerosis: Molecular Mechanisms of Neuronal Death and Protection

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Mutations in human Cu/Zn superoxide dismutase-1 (SOD) cause ~20% of cases of familial amyotrophic lateral sclerosis (FALS). We investigated the mechanism of mutant SOD-induced neuronal degeneration by expressing wild-type and mutant SODs in neuronal cells by means of infection with replication-deficient recombinant adenoviruses. Expression of two FALS-related mutant SODs (A4V and V148G) caused death of differentiated PC12 cells, superior cervical ganglion neurons, and hippocampal pyramidal neurons. Cell death included many features typical of apoptosis. Death could be prevented by copper (Cu^{2+}) chelators, Bcl-2, glutathione, vitamin E, and inhibitors of caspases. Mutant SOD-expressing PC12 cells had higher rates of superoxide (O_2^-) production under a variety of conditions. The results support the hypothesis that mutant SOD induced-neurodegeneration is associated with disturbances of neuronal free radical homeostasis.

Key words: familial amyotrophic lateral sclerosis; superoxide dismutase-1; apoptosis; recombinant adenovirus; neurodegeneration; oxidative stress

Amyotrophic lateral sclerosis (ALS) is a fatal progressive paralytic disorder of unknown cause involving motor neurons of the brain and spinal cord. Approximately 10–15% of ALS cases are autosomal dominantly inherited. More than 30 sites for mutations in a ubiquitously occurring cytoplasmic enzyme, Cu/Zn superoxide dismutase-1 (SOD), have been identified in ~20% of patients with dominantly inherited familial ALS (FALS) (Brown, 1995). Identification of these mutations suggested that free radicals play a critical role in the pathogenesis of the disease (Deng et al., 1993). It remained unclear, however, whether SOD mutation produced motor neuron death because of loss of SOD enzymatic activity or gain of an adverse function. Subsequent studies involving the transduction of mutant SOD genes into non-neuronal cells (Borchelt et al., 1994) and involving transgenic mice (Gurney et al., 1994; Ripps et al., 1995) demonstrated no correlation between SOD activity and the frequency or severity of the disease, suggesting that mutant SOD does not cause FALS because of a deficiency in SOD activity. The latter hypothesis was also supported by studies demonstrating the viability of motor neurons in SOD knock-out mice (Reaume et al., 1996).

These observations suggested that mutant SOD produces motor neuron death because of gain of a new adverse function or enhancement of a nondismutase activity of SOD that is normally present. The latter hypotheses were bolstered by experiments in yeast as well as in a continuous rat nigral cell line that had been permanently transfected with wild-type or mutant SOD cDNA (Rabizadeh et al., 1995). Expression of FALS-associated SOD mutants promoted rat nigral cell death after serum withdrawal or application of a Ca^{2+} ionophore, despite the fact that they had significant SOD enzyme activity. In contrast, overexpressed wild-type SOD inhibited cell death. A potential drawback of the latter studies is that the permanently transfected cells may have manifested additional phenotypes besides the one specifically associated with mutant SOD, as a result of selection.

The new or enhanced function of FALS-linked mutant SODs remains unclear. Beckman and colleagues (Beckman et al., 1993) have proposed that peroxynitrite, a product of superoxide (O_2^-) and nitric oxide (NO), reacts with the Cu^{2+} of mutant SODs, producing nitronium ions, which lead to nitration of proteins and subsequent neurotoxicity. This hypothesis was supported by studies demonstrating that motor neurons of ALS patients exhibit increased immunoreactivity for nitrotyrosine (Abe et al., 1995; Chou et al., 1996). An alternative hypothesis was proposed by Wiedau-Pazos et al. (1996) and Yim et al. (1996), who reported enhanced peroxidase activity of mutant SOD compared with wild type on the basis of spin trap studies. The enhanced peroxidase activity may increase production of hydroxyl radicals, which could damage neurons. These *in vitro* spin trap studies, however, may not accurately reflect the situation within neural cells expressing mutant SOD. Wong et al. (1995) recently proposed that the FALS-linked mutant SOD failed to bind or shield copper (Cu^{2+}) as effectively as the wild-type enzyme. This change could lead to an enhancement of Cu^{2+}-catalyzed oxidative reactions.

In this report, we describe a unique approach for studying the mechanisms underlying neuronal death induced by mutant SOD. We used replication-deficient recombinant adenoviruses (AdVs) to deliver and express human wild-type or mutant SOD genes into primary neurons as well as differentiated rat pheochromocytoma cells (PC12 cells). We then determined the effect of this overexpression on cell viability. Using an imaging assay (Bindokas et al., 1996), we were able to determine the O_2^- content of single cells. Our results indicate that expression of mutant SOD

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induces neural cell death that bears the hallmarks of apoptosis, is sensitive to metal chelators, antioxidants, and antiapoptotic agents, and is associated with abnormalities in free radical production.

MATERIALS AND METHODS

Cell culture. Human embryonic kidney (HEK) 293 and baby hamster kidney (BHK-21) monolayer cell cultures were grown in DMEM supplemented with 10% fetal bovine serum and 0.01% gentamycin (Life Technologies, Gaithersburg, MD). Rat pheochromocytoma PC12 cells were plated on poly-L-lysine-coated glass coverslips at a density of 10,000 cells per coverslip in DMEM supplemented with 10% bovine calf serum and 10 μg/ml penicillin/streptomycin (Sigma, St. Louis, MO). Differentiation of PC12 cells was induced within 24 hr after the addition of DMEM containing 100 ng/ml of nerve growth factor (NGF) (Collaborative Biomedical Products, Inc.) and no serum. Seven days later, cells were infected with recombinant AdVs as described below. Primary sympathetic neurons were isolated from superior cervical ganglia of 3- to 5-d-old Holtzman rats by previously described methods (Jordan et al., 1995). Dissociated cells were maintained on coverslips in L-15 medium with 1 μg/ml NGF and 5% rat serum (Life Technologies, Grand Island, NY). E17 hippocampal neuronal cultures were isolated according to the method of Banker (1980) with some modifications (Abele et al., 1990; Scholz and Miller, 1991) and grown on coverslips. Rat cortical astrocytes were cultured following the method of Landis and Weinstein (1983). The astrocytes were used for virus infection when they had reached confluency in DMEM with 5% horse serum and maintained in DMEM with N2.1 in the presence of AraC to prevent cell proliferation.

Preparation of recombinant replication-deficient AdVs expressing wild-type or mutant SOD. The preparation of AdVs expressing wild-type SOD has been described previously (Jordan et al., 1995). The SOD cDNA was placed downstream from elongation factor 1-α promotor (EF-1α) and upstream of cellular heavy chain enhancer (4F2) and the bovine growth hormone polyadenylation site. We also prepared recombinant AdVs expressing SOD that carry a mutation in exon 1 changing alanine to valine at amino acid position 4 (the most common FALS-linked mutation in North America) (Deng et al., 1993) and one with a mutation of valine to glycine at amino acid position 148 in exon 5. Each of the mutations was separately engineered into a shuttle vector, pAdKN (Jordan et al., 1995) as follows. Briefly, a *Pst*I–*Pst*I fragment of SOD cDNA was first cloned into the *Pst*I site of pTZ18R phagemid (Pharmacia, Piscataway, NJ) and then mutated using a Bio-Rad (Hercules, CA) *in vitro* mutagenesis kit with the following mutant oligonucleotide primers: 5′-CAC GCA CAC GAC CTT CGT CGC-3′ (Ala→Val) and 5′-GAT CCC AAT TCC ACC ACA AGC-3′ (Val→Gly). A blunt-ended *Pst*I–*Nhe*I fragment of mutant SOD cDNA (*Pst*I–*Nhe*I) was then cloned into the *Eco*RV site of pAdKN to generate pAdKN.SODA4V and pAdKN.SODV148G. These shuttle vectors were then used to generate recombinant replication-deficient wild-type AdSODWT and two mutants, AdSODA4V and AdSODV148G, following previously described methods (Jordan et al., 1995). Viruses used for neural cell infections were plaque-purified three times to isolate a homogeneous population and sequenced to confirm the mutations in the SOD gene. Viruses were gradient-purified by CsCl isopycnic centrifugation, dialyzed against HEPES-buffered saline (in mM: 10 HEPES, 140 NaCl, and 2 $MgCl_2$, pH 7.5) containing 10% glycerol, stored at −70°C in small aliquots, and titered by plaque assay. In some experiments, we infected cells with an AdV that expresses calbindin D_{28k} (AdCABP virus) (Chard et al., 1995) and, as an additional control, with AdLacZ virus (Barr et al., 1994).

Recombinant virus infections and treatment with drugs. BHK-21 and HEK 293 cells were infected for 1 hr with recombinant AdVs in a sufficient volume of postinfection media (culture media containing 2% serum) to just cover the cells, washed once, and then incubated with the postinfection media. An aliquot (1–10 μl) of purified virus was added to the medium to achieve a multiplicity of infection (MOI) of 100–1000 plaque-forming units per cell. The supernatant was then removed and replaced with postinfection medium. In the case of PC12 cells, 7 d after differentiation in DMEM with NGF, the medium was removed and replaced with virus (in the same medium) at a concentration similar to that noted above. Two hours later, the supernatant was removed, and new medium with or without drug was added. In the case of primary sympathetic neurons, the cultures were infected after 3 d in culture. In some experiments, NGF was withdrawn from neurons 3 d after infection, and a 1:1000 dilution of anti-NGF serum (Sigma) was added. Coverslips containing hippocampal neurons were removed from the glial feeder plate after 5–7 d in culture and placed face up in a 60 mm tissue culture dish containing 2 ml of glial-conditioned medium and virus at a dosage noted above. Two hours later, coverslips were transferred back into the original plates, facing down as before, and incubated for up to 8 d.

Drugs were added to cells immediately after infection and replenished in fresh media every 3 d. The drugs that were used included: tetraethylenepentamine (TEPA) (50 μM; Sigma), a Cu^{2+} chelator; EUK-8 (100 nM; Eukarion, Inc., Bedford, MA), which is an SOD mimic, and an analog of the latter with improved catalase activity, EUK-134 (100 nM; Eukarion); benzyloxycarbonyl-Val-Ala-Asp(*O*-methyl)-fluoromethylketone (ZVAD-FMK) (50 μM; Enzyme Systems Products, Inc., Dublin, CA) and acetyl-Tyr-Val-Ala-Asp-chloromethylketone (Ac-YVAD-CMK) (100 μM; Bachem Bioscience, Inc., Torrance, CA), which are caspase inhibitors; *N*ω-nitro-L-arginine (1 mM; Sigma), a nitric oxide synthase (NOS) inhibitor; glutathione ethyl ester (1 mM; Sigma); vitamin E (100 μM; Sigma); and *S*-nitroso-*N*-penicillamine (SNP) (10 μM; Sigma). The Ac-YVAD-CMK and ZVAD-FMK were stored in aliquots in DMSO at −80°C and diluted before use.

For experiments involving Bcl-2, PC12 cells were transduced with a pCMV7 retrovirus vector containing cDNA encoding Bcl-2 or with pCMV7 without an insert and then selected with geneticin, as described previously (Wagner et al., 1993); these retrovirus vectors were kindly provided by Dr. Nissim Hay (University of Chicago). Expression of the transduced Bcl-2 was confirmed by immunohistochemical staining and Western blot (data not shown). The control transduced PC12 cells and the PC12 cells expressing Bcl-2 cells were infected with recombinant AdVs to test the protective effect of Bcl-2.

Western blot analysis. BHK-21 cells were either mock-infected or infected with AdLacZ, AdSODWT, AdSODA4V, or AdSODV148G viruses at an MOI of 10 and incubated for 72 hr. Cells were scraped, washed with cold PBS, swollen for 15 min on ice with hypotonic buffer (in mM: 10 HEPES, 10 KCl, and 1 dithiothreitol, pH 7.5) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 500 U/ml aprotinin, and 1 μg/ml leupeptin), and then lysed for 15 min with 1.0% Nonidet P-40. Cytoplasmic proteins were obtained by centrifugation at 12,000 rpm for 15 min at 4°C. The proteins (50 μg) were separated on 12.5% SDS-PAGE. Separated proteins were transferred onto nitrocellulose paper and immunostained with horseradish peroxidase-conjugated anti-SOD polyclonal antibody (The Binding Site, catalog #PP077) using an enhanced chemiluminescence (ECL) Western blotting detection system (Amersham, Arlington Heights, IL). Nitration of proteins was analyzed on the protein extracts prepared from differentiated PC12 cells. Cytoplasmic extracts were prepared in a similar manner as described above. Western blot of proteins (100 μg) was probed with rabbit anti-nitrotyrosine polyclonal antibody (Upstate Biotechnology, Lake Placid, NY) and detected by using horseradish peroxidase-conjugated donkey anti-rabbit antibody (Amersham) followed by an ECL Western blotting detection system.

Immunocytochemical analysis. Neurons and PC12 cells on coverslips were fixed at 37°C for 15 min with 4% paraformaldehyde, washed three times with 0.1 M PBS, and permeabilized with 0.1% Triton X-100 in PBS for 2.5 min. Cells were treated at 25°C for 1 hr with blocking medium (0.1% Tween 20, 4% bovine serum albumin, and 0.1 M PBS) and then incubated at 4°C overnight with a 1:300 dilution of murine anti-human SOD monoclonal antibody (Sigma). Immunoreacted primary antibody was detected by a 1:500 dilution of anti-mouse IgG antibody–alkaline phosphatase (Boehringer Mannheim, Indianapolis, IN) and X-phosphate as a chromogen in blocking medium (Jackson ImmunoResearch, West Grove, PA).

Cell viability. The effect of wild-type or FALS-linked mutant SOD gene expression on viability of cells was determined using fluorescein diacetate (Rotman and Papermaster, 1966), propidium iodide (Molecular Probes, Inc., Eugene, OR) (Krishan, 1975) double staining. The stained cells were examined immediately under a fluorescence microscope (Leitz, Wetzlar, Germany). Cells with red nuclei staining for propidium iodide represent dead cells, whereas cells positive for fluorescein staining represent living cells. Five random microscopic fields were counted for each coverslip, and a total of 15 fields were examined for each drug treatment. In addition, Hoechst 33342 (Molecular Probes) (Telford et al., 1992) and terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) stain (Gavrieli et al., 1992) were used to assess chromatin condensation and DNA nicking as published (Prehn et al., 1996).

The percentage of cells surviving at the time indicated in the text was calculated after infection with mutant SOD as the average number of living cells after a particular treatment compared with the number of viable cells without treatment from at least three different experiments. In the case of sympathetic neurons, morphological changes were used as the criterion for viability and were assessed at different times after infection.

Measurement of cellular superoxide production. O_2^- generation in individual cells was monitored using hydroethidine (HEt) as described previously (Bindokas et al., 1996). In this assay, HEt (Molecular Probes) is oxidized by O_2^- anions to red fluorescent ethidium. Ethidium fluorescence was measured over time using digital-imaging microfluorimetry and Fluor software (Universal Imaging, Inc., West Chester, PA). Regions of interest were positioned over cell somas in a phase-contrast image of the field, background was subtracted, dye (3 μM) was added, and fluorescence (in nm: excitation, 510/25; dichroic mirror, 580; emission, 590; Nikon) increase was recorded in images (16 frame average) taken every 10 sec over a 7 min period in each treatment. After collection of basal rates, the solution was replaced with the mitochondrial uncoupler carbonylcyanide-*p*-(trifluoromethoxy)phenyl hydrazone (FCCP; Sigma; 1 μM; +HEt) and, finally, with one containing 1 mM H_2O_2 (+HEt). Linear regressions were fit to the rises obtained during each treatment for each cell and used as the measure of O_2^- production. Statistical comparisons of slopes were made with the Kolmogorov–Smirnov test.

Total SOD activity in PC12 cell lysates was determined either by use of a colorimetric assay kit (Calbiochem, San Diego, CA) or by direct measurement of O_2^- disproportionation (Marklund, 1976). Preparation of cell lysates and protein quantitation were performed as described above for Western blot analysis. SOD activity in equal quantities of total lysate protein was determined in triplicate for two to three different cell preparations. SOD activity in wild-type SOD- and mutant SOD-expressing cells was normalized to that of mock-infected controls.

RESULTS

Overexpression of wild-type and FALS-associated mutant SOD using replication-deficient adenoviruses

We prepared recombinant AdVs with wild-type and mutant SODs as vectors for gene delivery into primary neural cells (Fig. 1*A*). The presence of the mutation in the SOD cDNA was confirmed by PCR and sequencing (data not shown). We then tested whether expression of the SOD transgene resulted from the AdV infection. A Western blot of BHK-21 cells demonstrated the presence of an immunostained protein product in the mock-infected (Fig. 1*B, lane 1*) as well as the AdV-infected cells (Fig. 1*B, lanes 2–5*), corresponding to the electrophoretic mobility of rodent SOD. This immunostaining was a result of cross-reactivity of the anti-human SOD polyclonal antibody with endogenous rodent SOD. An additional immunostained band of slower electrophoretic mobility was present in extracts from cells infected with AdSODWT, AdSODA4V, and AdSODV148G viruses (Fig. 1*B, lanes 3–5*, respectively), corresponding to the electrophoretic mobility of human SOD. The levels of human SOD that were expressed in these cells were approximately similar to those of endogenous SOD. A previous study has demonstrated functional activity of the AdSODWT virus (Jordan et al., 1995). Total SOD activity at 3 d after infection was similar in mock-infected PC12 cells as well as cells infected with AdSODWT and AdSODV148G virus [relative activities were 100, 103 ± 11, and 103 ± 3% for mock, wild-type (WT), and V148G expression, respectively].

Effects of overexpression of mutant SODs on neural cell viability

We determined the effects of expression of wild-type and mutant SODs on differentiated PC12 cells, because they are frequently used as a model system for differentiated neural cells. Under our experimental conditions, infection with AdSODWT led to expression in ~60 ± 8% (n = 7) of the cells, as demonstrated by immunopositive staining with human SOD-specific antiserum (Fig. 2*B*). Comparable results were obtained after AdSODV148G infection (Fig. 2*C*) and after infection of primary neuronal cells (data not shown; see below). We were unable to test for expression of the SODA4V mutant transgene with immunohistochemical stains because of the lack of an available antibody that can differentiate the A4V mutant SOD from endogenous SOD; expression of this mutant was verified by Western blot (see above).

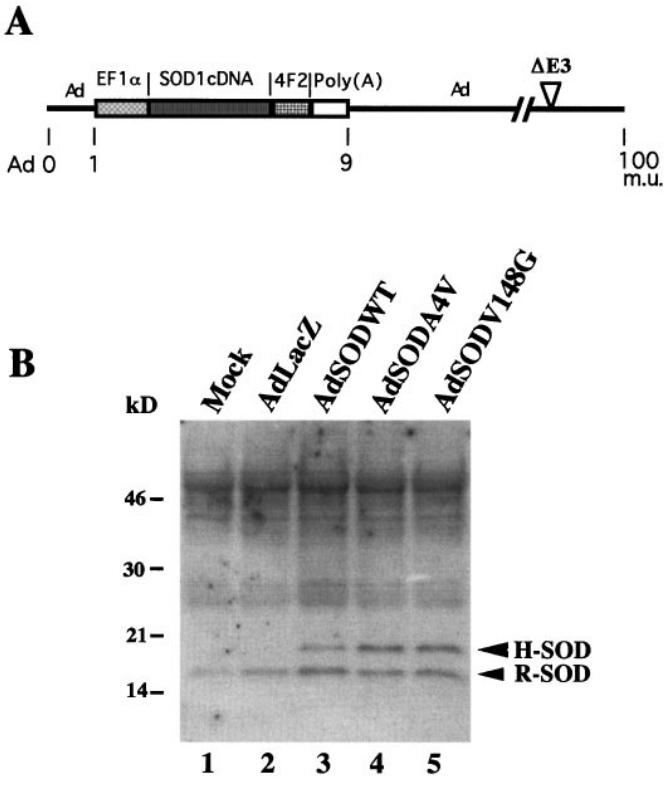

Figure 1. *A*, Schematic representation of the AdV-expressing wild-type or mutant SOD. The virus contains a deletion in the early region 3 (Δ*E3*), replacement of early region 1 with EF-1α, Cu/Zn superoxide dismutase-1 cDNA (*SOD1cDNA*), cellular 4F2 heavy chain enhancer (*4F2*), bovine growth hormone polyadenylation site [*poly*(*A*)], and adenovirus type 5 map units (*m.u.*). The gene lengths shown are not to scale. *B*, Western blot of BHK-21 cells after mock infection (*lane 1*) or infection with AdLacZ (*lane 2*), AdSODWT (*lane 3*), AdSODA4V (*lane 4*), or AdSODV148G virus (*lane 5*). The cells were lysed, and the lysates were then subjected to SDS-PAGE, blotted onto nitrocellulose, and incubated with an anti-SOD polyclonal antibody. There is evidence of immunostaining of a protein species corresponding to the electrophoretic mobility of rodent SOD (*R-SOD*) in all lanes and of human SOD (*H-SOD*) after infection with the wild-type and mutant SOD recombinant AdVs (*lanes 3–5*). The other higher molecular weight proteins that are immunostained are nonspecific.

We compared PC12 cell viability after infection with AdVs expressing mutant SODs with that seen after infection with a virus expressing wild-type SOD. Compared to mock-infected controls, approximately half of the PC12 cells died 5 d after infection with AdSODA4V and AdSODV148G (Fig. 3*A*). In contrast, there was no decline in survival of cells 5 d after infection with AdV expressing wild-type SOD (AdSODWT) when compared with mock-infected cells (Fig. 3*A*). It is also clear that cells that died and those expressing mutant SODs were the same population. Three days after infection ~60% of cells showed immunoreactivity for WT SOD or SODV148G. After 5 d, however, 54 ± 4% of cells were stained positive for WT SOD (n = 3), whereas only 14 ± 3% were now positive for SODV148G (n = 3).

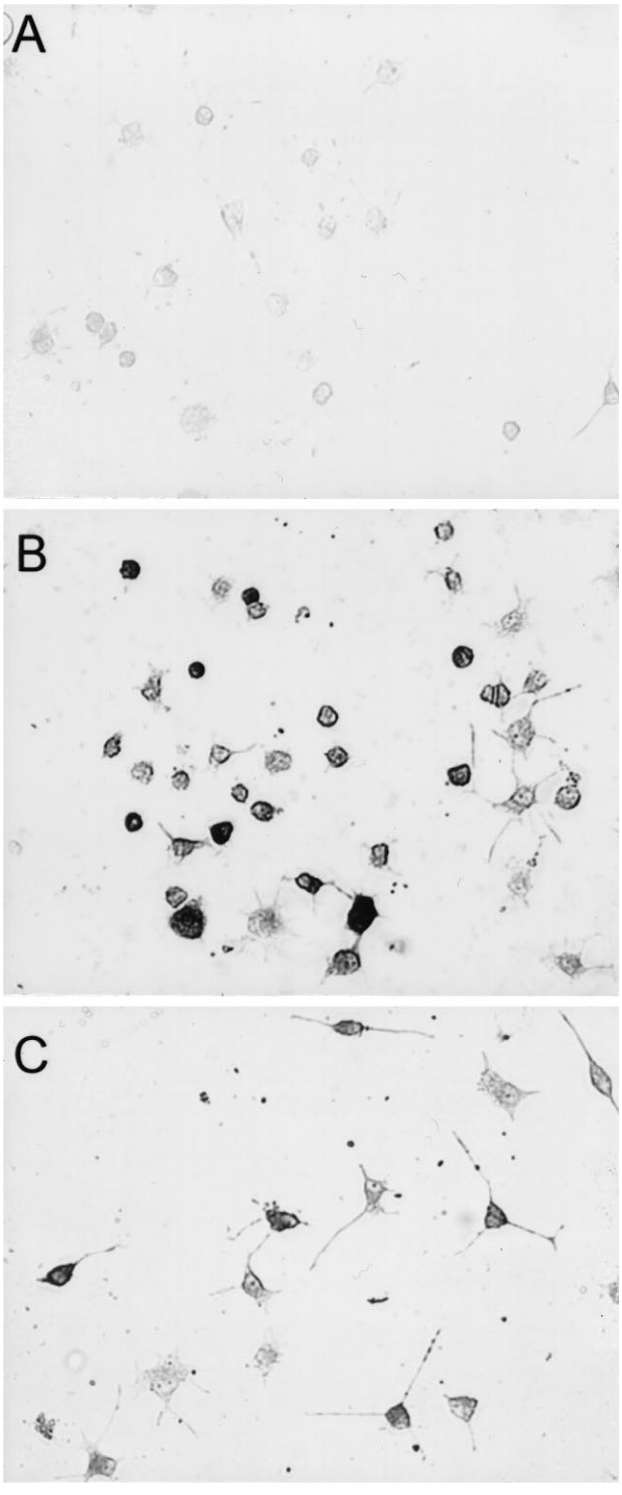

Figure 2. Immunohistochemical staining of PC12 cells (*A–C*) with anti-human SOD antibody after mock infection (*A*) or infection with AdSODWT (*B*) and AdSODV148G (*C*) viruses. Further details are given in Materials and Methods.

We then studied the effect of wild-type or mutant SOD expression on primary sympathetic neurons during normal conditions as well as during the added stress of growth factor withdrawal. As shown in Figure 3*B*, there was no decline in viability of primary neural cells grown in the presence of NGF after infection with AdSODWT virus. In contrast, AdSODV148G and AdSODA4V induced cell death, so that only 60 and 45% of cells, respectively, remained after 5 d (Fig. 3*B*). As expected, uninfected primary sympathetic neurons died after NGF withdrawal and application of anti-NGF antiserum, a procedure known to induce apoptosis of these cells (Fig. 3*C*) (Martin et al., 1988). Infection with AdSODWT virus 3 d before NGF withdrawal almost completely protected these cells from further cell death (Fig. 3*C*), consistent with our previously published studies (Jordan et al., 1995). In contrast, infection with AdSODV148G and AdSODA4V led to an enhancement of cell death (Fig. 3*C*).

We also examined the effect of mutant SOD expression on cultured hippocampal pyramidal neurons. Three days after infection there was little evidence of cell death (Fig. 3*D*). By 5 d cell death increased after infection with AdSODV148G and AdSODA4V compared with mock-infected cells or cells infected with AdSODWT (data not shown), and by 8 d there was a very robust decrease in the number of viable cells after infection with the viruses expressing mutant SODs compared with the mock infection or after infection with AdSODWT virus (Fig. 3*E*).

To examine whether the cell death induced by mutant SOD was specific for neural cells, we also tested the effect of the recombinant AdVs on primary astrocytes. In contrast to neurons, astrocytes proved to be resistant to the effects of SOD mutants (Fig. 3*F*). Only 5–6% of the cells died by 5 d after infection, which was not significantly different from that after expression of WT SOD.

Characteristics of mutant SOD-induced neural cell death

We examined cells dying after expression of mutant SOD for evidence of apoptosis (Figs. 4, 5). Apoptotic changes were frequently found in these and detectable in many microscope fields examined; in contrast, these changes were rarely seen in cells infected with AdSODWT. Dying PC12 cells (Fig. 4), hippocampal pyramidal neurons (Fig. 5), or sympathetic neurons (data not shown) exhibited histological and morphological features of apoptosis, including shrunken cell soma, chromatin condensation (assessed using the Hoechst 33342–fluorescent diacetate–propidium iodide triple staining), and DNA nicking (assessed using TUNEL staining). As a comparison, we also performed experiments involving the death of differentiated PC12 cells and sympathetic neurons induced by removal of NGF (Martin et al., 1988). Cells dying after growth factor withdrawal showed features similar to those of the same cell types dying after mutant SOD expression (Fig. 4*E*).

In summary, we observed similar toxic effects of mutant SODs in differentiated PC12 cells and primary hippocampal and primary sympathetic neurons. For this reason, and because of the greater ease in performing studies with differentiated PC12 cells, we used these cells for future characterization of mutant SOD-induced cell death.

Inhibition of SOD mutant-induced neural cell death

To clarify the mechanism(s) by which mutant SODs induced death of PC12 cells, the cells were treated with different agents after recombinant AdV infection (Fig. 6*A,B*). These results were compared with the effects of the same agents on the death of PC12 cells caused by removal of NGF (Fig. 6*C*). We tested the effect of ZVAD-FMK and Ac-YVAD-CMK, two irreversible inhibitors of interleukin 1β-converting enzyme-like proteases (caspases) that have been implicated in apoptosis (Troy et al., 1996b). Ac-YVAD-CMK inhibited PC12 cell death 5 d after infection with AdSODV148G virus (Fig. 6*A*), although the drug

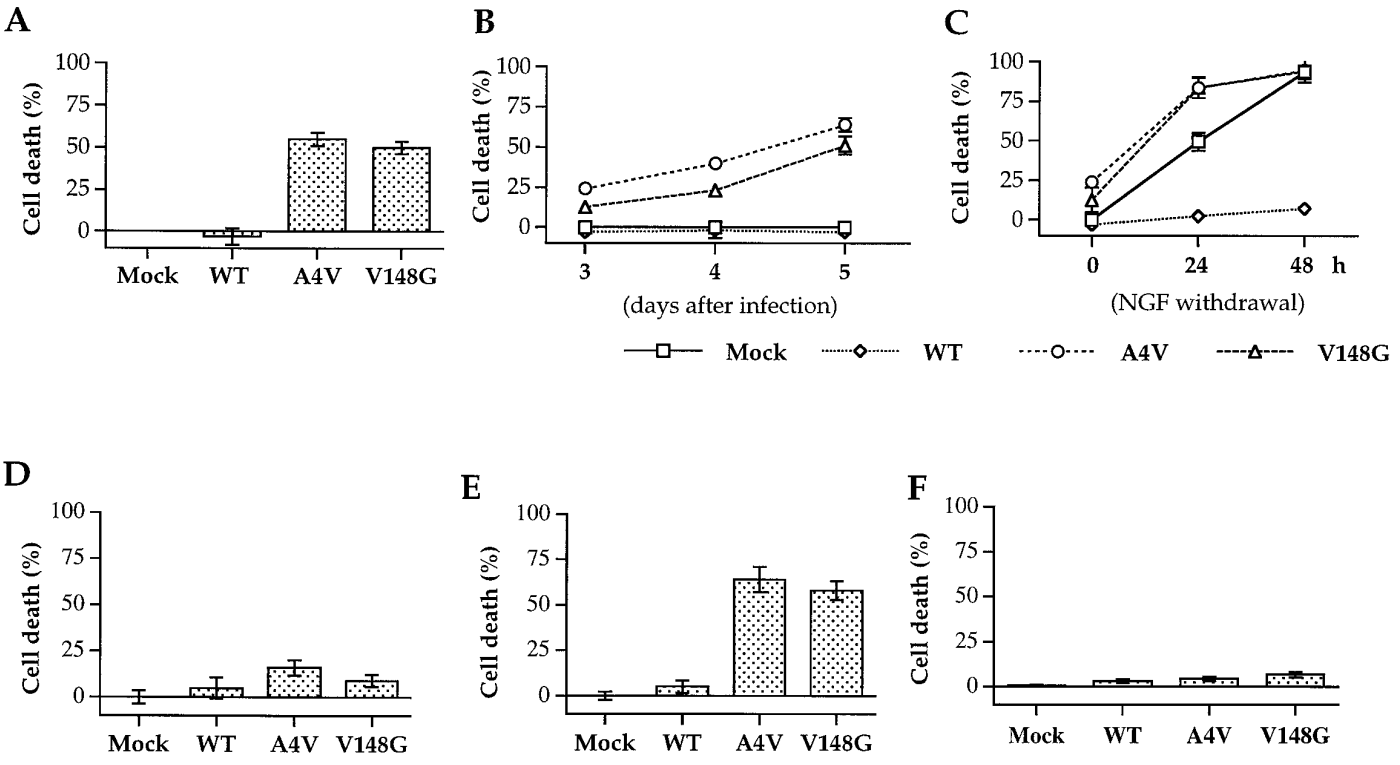

Figure 3. Cell death of differentiated PC12 cells 5 d after infection (*A*), primary sympathetic neurons 3–5 d after infection in the presence (*B*) or absence (*C*) of NGF, primary hippocampal neurons [3 (*D*) or 8 (*E*) d after infection], and astrocytes 5 d after infection (*F*) with mock or AdSODWT, AdSODA4V, or AdSODV148G virus. Note that time shown on the *abscissa* of *D* refers to the time after withdrawal of NGF from the sympathetic neurons, which was begun 3 d after infection.

had only a small effect after infection with AdSODA4V virus (Fig. 6*B*). ZVAD-FMK protection of cells infected with either of the two AdVs expressing mutant SODs was more modest but again was greater in the case of expression of the V148G mutant (Fig. 6*A,B*). Because Bcl-2 has been found to be protective in many examples of apoptosis (Hockenbery et al., 1993), we tested PC12 cells that overexpressed Bcl-2 as a result of retroviral transduction (see Materials and Methods). Bcl-2 overexpression had a significant effect on cell viability after AdSODA4V and AdSODV148G virus infection, protecting 60% or more of the cells from death (Fig. 6*A–C*). Both of the caspase inhibitors and Bcl-2 overexpression also protected PC12 cells from death after NGF removal (Fig. 6*C*). Because increased $[Ca^{2+}]_i$ frequently accompanies cell death, and because neurons overexpressing some Ca^{2+}-binding proteins have been shown to be selectively spared in ALS (Ince et al., 1993; Ho et al., 1996), we tested the effect of overexpression of a Ca^{2+}-binding protein, calbindin D_{28K} (Chard et al., 1995). Pretreatment of PC12 cells with an AdV-expressing calbindin D_{28k} (AdCABP) produced a modest protective effect after a subsequent infection with AdV expressing either of the mutant SODs (Fig. 6*A,B*). Immunocytochemical staining confirmed calbindin D_{28K} expression in 60 ± 5% (n = 3) of the infected cells (data not shown).

The most dramatic inhibition of PC12 cell death after infection with AdSODV148G and AdSODA4V viruses was obtained after treatment with TEPA, a Cu^{2+} chelator (Fig. 6*A,B*). Inhibition was nearly complete in the case of cells dying after infection with AdSODV148G. In contrast, there was little effect of TEPA on cells dying after NGF withdrawal (Fig. 6*C*). The substantial inhibition by TEPA of cell death after mutant SOD expression suggests that death is induced by an active process that is disrupted by TEPA, rather than by a mere deficiency in SOD activity. The relative lack of a protective effect of TEPA on death induced by NGF withdrawal suggests that the initial stages of the pathway involving the mutant SOD-induced apoptosis differ from those resulting from growth factor withdrawal. To determine whether SOD activity played a role in the protective effects of TEPA against SOD mutant toxicity, we determined SOD activity in these cells. Total SOD activity was similar in mock-infected, WT, and AdSODV148G-expressing PC12 cells as described earlier. TEPA treatment significantly decreased total SOD activity by ~35% in all cases.

Because diverse forms of cell death have been associated with free radicals, we treated dying PC12 cells with glutathione, vitamin E, EUK-8, and EUK-134. A significant inhibition of cell death induced by expression of SODV148G was observed after treatment with the antioxidant compounds glutathione and vitamin E (Fig. 6*A*). Cell death induced by NGF withdrawal was also inhibited by vitamin E, but there was no significant inhibition by glutathione. Both EUK-8 and EUK-134, which are SOD mimics that also have catalase activity (Bruce et al., 1996), inhibited PC12 cell death resulting from mutant SOD expression or NGF withdrawal (Fig. 6*A–C*). A greater effect was in protecting cells infected with AdSODV148G than with AdSODA4V. EUK-134 (which has higher catalase activity) was more effective in protecting cells than EUK-8. The effects of the glutathione, vitamin E, and SOD mimics suggest a role for reactive oxygen species. This possibility was further investigated using an imaging paradigm (see below).

Beckman et al. (1993) have proposed that FALS-associated mutant SOD leads to an increase in the reaction of peroxynitrite

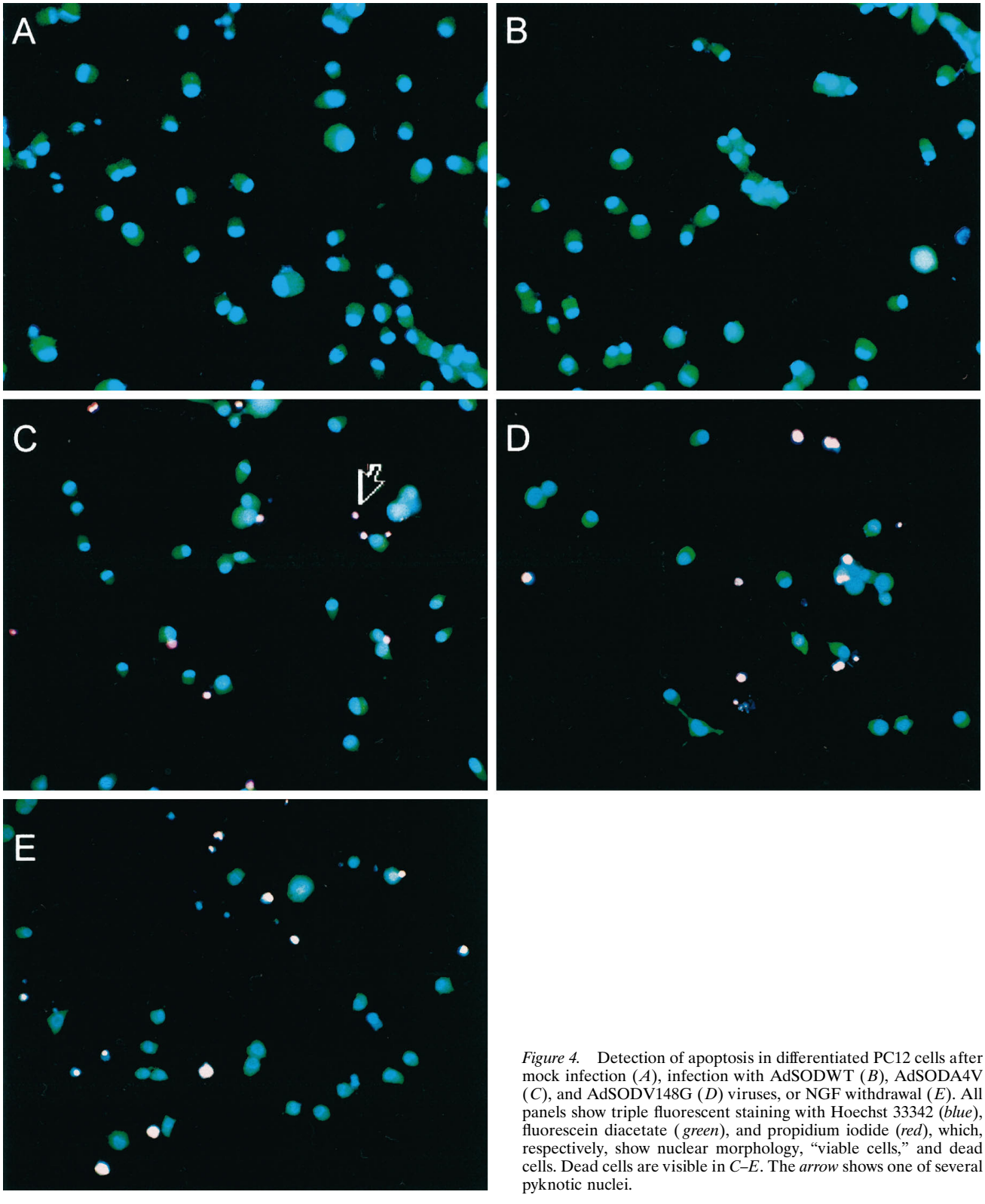

Figure 4. Detection of apoptosis in differentiated PC12 cells after mock infection (*A*), infection with AdSODWT (*B*), AdSODA4V (*C*), and AdSODV148G (*D*) viruses, or NGF withdrawal (*E*). All panels show triple fluorescent staining with Hoechst 33342 (*blue*), fluorescein diacetate (*green*), and propidium iodide (*red*), which, respectively, show nuclear morphology, "viable cells," and dead cells. Dead cells are visible in *C–E*. The *arrow* shows one of several pyknotic nuclei.

with SOD, and that the subsequent nitration of proteins damages cells. To examine this further, we treated PC12 cells with *N*ω-nitro-L-arginine, an NOS inhibitor, at the time of infection. Minimal protection was observed after infection with AdSODV148G or AdSODA4V or after NGF withdrawal (Fig. 6*A–C*), suggesting that NO does not play a significant role in cell death in these cases. To examine this issue further, we immunostained PC12 cells with an anti-nitrotyrosine antibody after infection with AdVs expressing mutant SODs. We found no difference in the staining intensity between the latter cells and cells infected with mock or wild-type virus (data not shown). Similarly, Western blot analysis using anti-nitrotyrosine antibody of the proteins from the

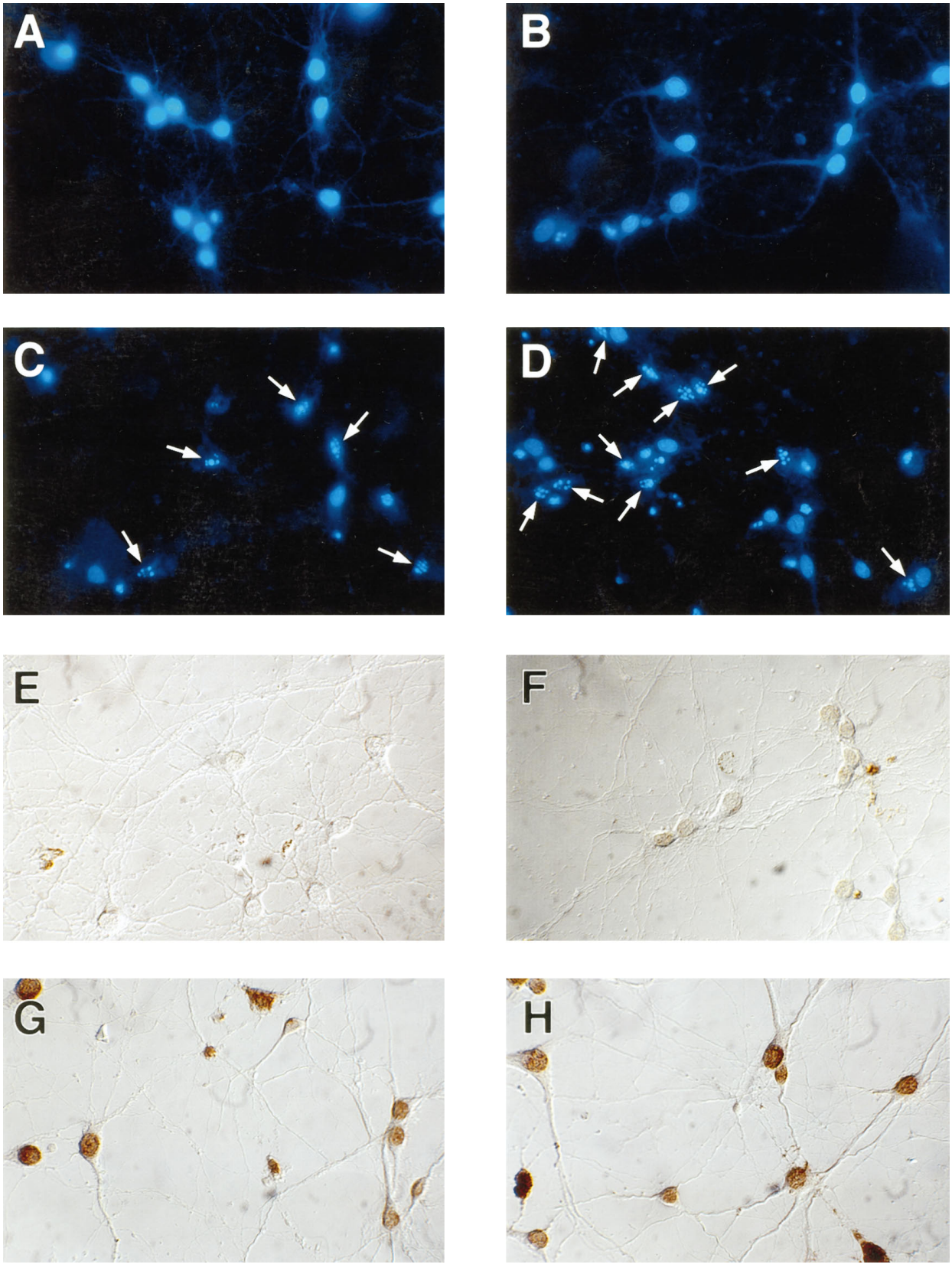

Figure 5. Detection of apoptosis in primary hippocampal neurons with Hoechst 33342 staining (*A–D*) or TUNEL staining (*E–H*) that were mock-infected (*A, E*) or infected with AdSODWT (*B, F*), AdSODV148G (*C, G*), or AdSODA4V virus (*D, H*). *Arrows* indicate nuclei with chromatin condensation and fragmentation.

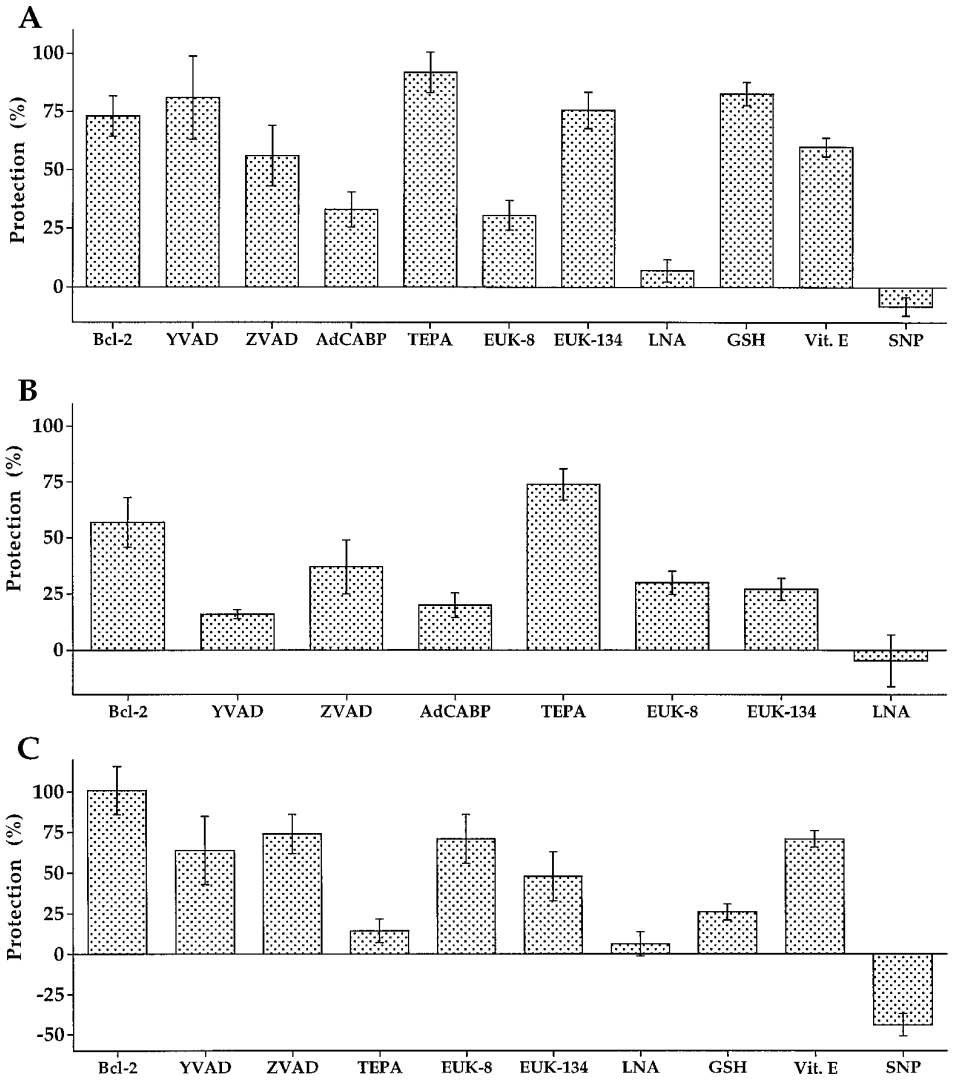

Figure 6. Protective effect of varying agents on cell death of differentiated PC12 cells induced by AdSODV148G (*A*), AdSODA4V (*B*), or after NGF withdrawal (*C*). Drug treatment application of Ac-YVAD-CMK and ZVAD-FMK (caspase inhibitors), TEPA (a Cu^{2+} chelator), EUK-8 and EUK-134 (SOD mimics), *N*ω-nitro-L-arginine (*LNA*; an NOS inhibitor), glutathione (*GSH*), vitamin E, and *S*-nitroso-*N*-penicillamine (*SNP*). Calbindin D_{28k} was overexpressed as a result of Ad-CABP infection, and Bcl-2 was overexpressed as described in Materials and Methods. Protection is the percent increase in cell viability above that seen after treatment with the same mutant virus without additional treatments (*A, B*) or above that seen after NGF withdrawal without additional treatments (*C*). Values are expressed as mean ± SEM of three experiments. There was no significant decline in the viability of control cells during the course of rescue experiments.

cells transduced with AdSODV148G failed to show evidence of nitration (data not shown). To address the issue of whether the lack of evidence for the nitration of proteins is because of the low levels of NOS in the differentiated PC12 cells, we treated the cells with the NO generator SNP. Although SNP enhanced cell death by 8 ± 4% in the cells expressing SODV148G and by 44 ± 7% after NGF withdrawal, it had no effect on the mock-infected cells or cells infected with AdSODWT.

Superoxide production in PC12 cells

Because our pharmacological studies suggested that reactive oxygen species were associated with cell death induced by mutant SODs, we analyzed differentiated PC12 cells with a single-cell microfluorimetry assay that uses the selective oxidation of HEt by O_2^- (Bindokas et al., 1996). Rates of O_2^- production were low in differentiated PC12 cells. Based on measurements from >100 cells, the basal O_2^- production rate was 1.31 ± 0.08 fluorescent

Table 1. HEt oxidation rates in PC12 cells 3 d after infecrtion with various AdVs

Infection	Basal Slope[a] (FIU/min)	n	Sig*	FCCP	n	Sig	H_2O_2	n	Sig
Mock	1.31 ± 0.09	147	a	2.08 ± 0.13	147	a	3.61 ± 0.18	147	a
AdLacZ	1.21 ± 0.08	123	a	2.08 ± 0.12	123	a	3.73 ± 0.19	123	a
AdSODWT	1.33 ± 0.09	134	a	2.25 ± 0.14	134	a	4.07 ± 0.20	134	a,b
AdSODV148G	1.49 ± 0.09	114	b	2.54 ± 0.16	114	b	4.69 ± 0.27	114	b,c
AdSODA4V	1.44 ± 0.1	115	a	2.35 ± 0.15	115	a	5.00 ± 0.26	115	c

[a]HEt is oxidized to ethidium by O_2^-, and the rate of Et fluorescence increase is expressed as the change in fluorescence over time (slope). Data represent mean ± SEM.
*Treatments with the same lowercase letters are not significantly different within each column ($p > 0.05$; Kolmogorov–Smirnov test).

units (FlU)/min (Table 1). Rates increased slightly in PC12 cells after addition of the mitochondrial uncoupler FCCP, consistent with the electron transport chain being one source of O_2^- generation (Bindokas et al., 1996). Addition of hydrogen peroxide (H_2O_2) significantly increased the oxidation of HEt, indicating an increase in O_2^-. This action was not attributable to a direct interaction of H_2O_2 with HEt (Bindokas et al., 1996) but a result of H_2O_2 inactivating or overwhelming other O_2^--scavenging pathways and possibly also enhancing the generation of O_2^-.

Expression of the two mutant AdSODs tended to increase basal rates of O_2^- production slightly, but only after V148G expression was this significantly higher than mock-treated cells ($p = 0.001$) (Table 1). The rate of O_2^- production after infection with the AdV-expressing wild-type SOD was nearly identical to that of mock-treated cells (1.33 ± 0.09), whereas there was a slightly lower rate (1.21 ± 0.08; $p = 0.48$) after infection with AdLacZ virus.

Application of FCCP (1 μM) increased the O_2^- production rates ~1.5 times to 2.08 ± 0.13 FlU/min in mock-treated cells and in cells infected with AdLacZ virus. There was a greater increase in slope for cells in which either of the mutant SODs were expressed. The highest rates were observed with the AdSODV148G mutant ($p < 0.05$), with less of an increase with AdSODA4V. These results were in contrast with those seen after expression of wild-type SOD, in which there was no significant change compared with controls.

Application of H_2O_2 further increased O_2^- production and amplified the trends observed. The largest increases occurred with cells expressing mutant SODV148G ($p = 0.04$ vs mock) and SODA4V ($p = 0.001$). Cells infected with AdSODWT were not significantly different from the mock-infected cells ($p = 0.23$). These data are consistent with an enhanced peroxidase activity for mutant SOD (Wiedau-Pazos et al., 1996; Yim et al., 1996).

DISCUSSION

The identification of mutations in SOD as a cause of FALS generated substantial excitement among neuroscientists, owing to the possibility that these findings might clarify the pathogenesis of not only FALS but sporadic ALS as well. Initially it was supposed that the FALS-associated mutant enzymes had inadequate SOD activity, thereby leading to an accumulation of O_2^- and other free radicals with the subsequent death of motor neurons. This hypothesis had to be modified, however, because a number of studies, including those involving FALS transgenic mice (Gurney et al., 1994; Ripps et al., 1995) and wild-type SOD knock-out mice (Reaume et al., 1996), suggested that FALS-associated mutant SODs induced neuronal damage by another mechanism rather than one involving impaired dismutase enzyme activity.

Past studies exploring the effect of mutant SODs *in vitro* have primarily involved yeast and non-neural cells. The only study that has investigated neural cells used a continuous rat nigral cell line that was permanently transfected with wild-type or mutant SOD cDNAs (Rabizadeh et al., 1995). A potential drawback of the latter studies is that continued growth of these permanently transfected cells may have led to compensatory changes attributable to expression of the deleterious gene. Experiments involving transient expression in primary cultured neurons have not previously been feasible because of the inefficiency of conventional methods for gene transduction into such cells. In the present report we circumvented this problem by using AdVs to deliver and express foreign genes efficiently in primary neuronal cells as well as a differentiated neural cell line.

Our studies demonstrated that mutant SODs induce the death of several types of differentiated neural cells. We found that cell death occurred in differentiated PC12 cells, primary sympathetic neurons, and primary hippocampal neurons. In contrast, there was little death of primary astrocytes, suggesting that neurons are more sensitive to the effects of mutant SOD. The greater cell death after the SODA4V expression compared with the SODV148G may be related to the greater toxicity of the former mutant, as suggested by the decreased survival of FALS patients who carry this mutation (Juneja et al., 1996). We found no evidence of increased cell death after transduction of these cells with wild-type SOD, demonstrating the differential effects of mutant versus wild-type SOD (Jordan et al., 1995).

The cell death that occurred after expression of mutant SODs had morphological features typical of apoptosis. This was also supported by our finding that antiapoptotic agents, such as Bcl-2 and caspase inhibitors, blocked cell death. These results as well as the finding of a robust protective effect of TEPA suggest that caspase inhibitors and TEPA should be tested for their ability to delay the onset or decrease the severity of the neurodegeneration in FALS transgenic mice. It may be that screening drugs that reverse the cell death in mutant SOD-expressing PC12 cells will provide a means to identify drugs that are effective in the treatment of FALS as well as sporadic ALS.

It should be noted, however, that the cell death data do not necessarily mean that neurons in FALS normally use an apoptotic mechanism of cell death during the disease state, because virtually any cell can die by apoptosis if prompted to do so by some adverse stimulus (Raff et al., 1994). Rather, the results support the idea that mutant SODs are perceived by these cells as noxious in some way and suggest that apoptotic mechanisms may also be involved in the neurodegeneration in FALS.

Oxidative damage and decreased free radical scavenging are both known to induce apoptotic cell death (Greenlund et al., 1995). In our studies, reactive oxygen species, especially O_2^-, appeared to be involved in the mutant SOD-induced cell death, because glutathione and SOD mimics tended to decrease this mortality. We also found that Bcl-2, which is known to affect free radical generation and cell viability similarly, protected the cells against the apoptosis induced by mutant SOD (Hockenbery et al., 1993). In addition, microfluorimetry demonstrated a slightly increased rate of O_2^- accumulation in PC12 cells after expression of mutant SODs, especially in cells undergoing oxidative stress; these data suggest that expression of mutant SOD increases the sensitivity to oxidative stress. Increased O_2^- can potentially lead to formation of additional reactive species, including hydroxyl radicals and peroxynitrite. Studies illustrating a beneficial effect of vitamin E on the clinical course of FALS transgenic mice (Gurney et al., 1996) and our results with vitamin E are also consistent with a role for oxidative stress in disease pathogenesis.

Our data regarding O_2^- suggest that the free radical content of cells expressing mutant SODs is disturbed, despite the expression of endogenous SOD. There are several possible explanations for our findings. A simple, slight decrease in dismutase activity could explain the increased O_2^- levels that generate additional reactive species. It is possible that the mutant SOD could interfere with the function of the wild-type SOD; i.e., there is a dominant negative effect; although there is some evidence for the latter effect in *Drosophila melanogaster* that carry a mutant SOD transgene (Phillips et al., 1995), there is little support for this activity in vertebrate cells (Borchelt et al., 1994). It may be that the SOD activity is unable to keep up with the enhanced generation of O_2^-

as a result, for example, of mitochondrial damage. Although SOD knock-out mice do not demonstrate anterior horn cell degeneration, they are more sensitive to injury, such as axotomy (Reaume et al., 1996). We believe, however, that it is unlikely that the mechanism of mutant SOD cell death involves a deficiency of SOD activity. Studies with PC12 cells have demonstrated that downregulation of SOD activity kills via an apoptotic pathway that is different from the apoptotic pathway that we found after mutant SOD expression. Thus, we found no protective effect of NOS inhibitors on the mutant SOD-induced apoptosis, whereas NOS inhibitors decrease cell death after SOD downregulation of PC12 cells (Troy et al., 1996a,b). These results cannot be explained by postulating a deficiency of SOD activity as the mechanism of cell death. Furthermore, total SOD activity in the WT and mutant SOD-expressing PC12 cells was similar, and TEPA treatment decreased this activity by 35% in all the cases.

It has been proposed that mutant SODs may possess a gain in function or enhancement of an existing toxic nondismutase function. Beckman and colleagues (1993) suggested that the mutant enzyme had an enhanced reactivity for peroxynitrite leading to an increase in the nitration of tyrosines. We tested this hypothesis by subjecting PC12 cells that had been infected by AdV-expressing mutant SOD to immunostaining and Western blot analysis using an antiserum that reacts with nitrotyrosine. We failed to find evidence of increased nitrotyrosine antigenicity in the virus-infected cells. We also investigated the potential importance of peroxynitrite in the mutant SOD-induced apoptosis by perturbing NOS activity leading to decreased or increased production of NO. Again, there was no evidence that decreasing NO synthesis affected cell viability or that increasing NO generation accelerated cell death; as mentioned above, these results contrast with experiments in PC12 cells in which downregulation of SOD activity produced NOS-dependent cell death (Troy et al., 1996a,b) and in which generation of NO enhanced cell death after NGF withdrawal (Fig. 6*C*) and suggest that mutant SOD may itself be a source for toxic radicals. These results fail to support a role for peroxynitrite and the subsequent nitration of proteins as a primary cause of cell death after expression of mutant SOD. The nitrotyrosine staining that has been found in motor neurons of ALS patients (Abe et al., 1995; Chou et al., 1996) may be a secondary and later change that follows a different primary injury to these cells. Thus, it may be that the O_2^- accumulation that we observed could lead, in particular cells, to the secondary production of a number of other free radical species, such as peroxynitrite. Our findings in mutant SOD-expressing cells are consistent with other possible mechanisms that have been proposed to explain the effects of mutant SODs. This includes an increase in peroxidase activity that is normally present in wild-type SOD and could be enhanced in the mutant form (Wiedau-Pazos et al., 1996; Yim et al., 1996) as well as a decrease in binding or shielding of the mutant enzyme to metals, which results in an increase in Cu^{2+}-catalyzed oxidative reactions.

Because all neuronal cells we tested were susceptible to the cell death of mutant SOD, the data do little to explain the selective vulnerability of motor neurons that occurs in FALS and ALS. It may be that the extraordinary metabolic activity of motor neurons puts them at a heightened risk with respect to damage from the free radical species that are generated as a result of the mutant SOD expression. Alternatively, there may be other factors present in the CNS that confer a selective vulnerability to motor neurons.

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