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Coincident thresholds of mutant protein for paralytic disease and protein aggregation caused by restrictively expressed superoxide dismutase cDNA

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Familial amyotrophic lateral sclerosis (FALS) has been modeled in transgenic mice by introducing mutated versions of human genomic DNA encompassing the entire gene for Cu,Zn superoxide dismutase (SOD1). In this setting, the transgene is expressed throughout the body and results in mice that faithfully recapitulate many pathological and behavioral aspects of FALS. By contrast, transgenic mice made by introducing recombinant vectors, encoding cDNA genes, that target mutant SOD1 expression to motor neurons, only, or astrocytes, only, do not develop disease. Here, we report that mice transgenic for human SOD1 cDNA with the G37R mutation, driven by the mouse prion promoter, develop motor neuron disease. In this model, expression of the transgene is highest in CNS (both neurons and astrocytes) and muscle. The gene was not expressed in cells of the macrophage lineage. Although the highest expressing hemizygous transgenic mice fail to develop disease by 20 months of age, mice homozygous for the transgene show typical ALS-like phenotypes as early as 7 months of age. Spinal cords and brain stems from homozygous animals with motor neuron disease were found to contain aggregated species of mutant SOD1. The establishment of this SOD1-G37R cDNA transgenic model indicates that expression of mutant SOD1 proteins in the neuromuscular unit is sufficient to cause motor neuron disease. The expression levels required to induce disease coincide with the levels required to induce the formation of SOD1 aggregates.

Keywords: FALS; Superoxide dismutase; Protein aggregation; Transgenic mice; Disease model

Introduction

Amyotrophic lateral sclerosis, characterized by muscle weakness, is caused by the death of specific populations of motor neurons (Rowland, 1994). Although most cases of ALS are of unknown etiology and deemed "sporadic", about 10% of cases are inherited as autosomal dominant familial illness (Siddique et al., 1991). In about one-fifth of familial patients and a few sporadic cases, disease has been linked to mutations in SOD1 (Rosen et al., 1993). To date, mutations at >60 different residues in SOD1 have been linked to familial ALS (www.alsod.org). With one exception, D90A, where both dominant and recessive (partially penetrant) modes of inheritance have been described, all SOD1 mutations cause disease in a dominant fashion. In cases where the D90A mutation shows a recessive and partially penetrant pattern of inheritance, it is thought that the recessive haplotype is due to a semi-dominant protective modifier located a short distance from, and in disequilibrium with, the SOD1 gene (Al Chalabi et al., 1998; Parton et al., 2002). Although SOD1 is the principal superoxide scavenging enzyme of the cytoplasm (Fridovich, 1986), substantial evidence has shown that SOD1 mutations lead to loss of motor neurons through a dose-dependent gain-of-property mechanism. Proposed mechanisms of toxicity include copper-mediated abnormal catalytic activity, disruptive aggregate formation, abnormal compartmentalization in mitochondria, and other properties (Cleveland and Rothstein, 2001). Although recent studies have demonstrated that copper-mediated chemistry is not crucial for toxicity (Subramaniam et al., 2002; Wang et al., 2003), the precise mechanisms by which different mutations in SOD1 gene eventually lead to the disease phenotype remain to be elucidated.

To model SOD1-linked FALS, transgenic mice have been generated by introducing human genomic DNA (Gn.SOD1) encoding disease-linked mutations, including the G37R, G85R, G93A, and H46R/H48Q mutations (Bruijn et al., 1997; Gurney et al., 1994; Wang et al., 2002b; Wong et al., 1995). Transgenic rats

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harboring human Gn.SOD1 with G93A or H46R mutations (Howland et al., 2002; Nagai et al., 2001) and mice harboring mutated versions of a mouse *sod1* genomic DNA fragment also develop ALS-like disease (Ripps et al., 1995). In all but the last of these studies, the transgene has been a 12-kb genomic fragment of human DNA that contains all coding exons and introns together with the 3′ and 5′ flanking regulatory regions. Included within this DNA fragment are signals sufficient to induce transcription and faithful translation of the genomic SOD1 transgene in all cell types in adult mice (Wong et al., 1995).

However, no transgenic mice engineered to express a mutant SOD1 complementary DNA (cDNA) gene in specific types of neural cells have ever been reported to develop ALS-like disease. The vectors used in these studies include an expression cassette containing promoter elements from the Thy-1 gene and driving the expression of G85R or G93A human SOD1 cDNAs in postnatal mouse neurons (Lino et al., 2002), a vector containing the neurofilament light chain promoter for neuron-specific expression of G37R cDNA (Pramatarova et al., 2001), and a vector containing the human glial fibrillary acidic protein (GFAP) promoter for astrocyte-specific expression of G85R cDNA (Gong et al., 2000). There are several possible explanations for the lack of symptoms in these transgenic mice. First, there may be specific spatial and temporal patterns of expression that are missing in the cDNA-based vectors. Second, the levels of expression may have been below the threshold required to induce disease in the relatively short lifespan of a mouse. Third, it is formally possible that elements in the genomic structure (for example, rare alternatively spliced variants, etc.) participate in the genesis of toxic protein. Lastly, it is formally possible, however unlikely, that mutant versions of human SOD1 mRNA possess toxic structural features.

To address these issues, we attempted to express mutant SOD1 cDNA with a vector derived from the mouse prion protein gene (MoPrP.Xho) (Borchelt et al., 1996a). With this vector system, mutant protein is expressed in both neurons and glia of the central nervous system (CNS), and in muscle at high levels. Although disease onset was late in mice hemizygous for the transgene, mating to homozygosity dramatically accelerated the onset of paralysis. In mice developing disease, aggregated forms of mutant SOD1 were detected in spinal cord. We conclude that expression of mutant SOD1 in the neuromuscular unit is sufficient to cause disease and that toxicity to motor neurons is likely to involve oligomeric forms of the mutant protein that are intermediates to or contained within aggregates.

Materials and methods

Transgenic mice

The SOD1-G37R cDNA was excised from the pEF.Bos vector (Borchelt et al., 1994) and inserted into the MoPrP.Xho vector (Borchelt et al., 1996a). The construct was verified by restriction endonuclease digestion and DNA sequence analysis, and then purified, linearized by *Not*I digestion, and gel purified. Before microinjection into B6/C3 F2 mouse embryos, the PrP construct encoding SOD1-G37R was mixed 1:1 with a previously described PrP construct encoding wild-type (WT) human presenilin 1 (hPS1) (Thinakaran et al., 1996). Offspring harboring the transgenes were identified by PCR. PS1 transgenes were detected following previously described PCR protocols (Thinakaran et al., 1996). PrP.SOD1-G37R transgenes were detected using a sense primer specific to human SOD1 (GTCGACAAGCATGGCCACGAAGG-CCGTGTGC) and an antisense primer specific to the 3′ untranslated region of the MoPrP.Xho vector (GTGGATACCC-CCTCCCCCAGCCTAGACC). Mice harboring fragments of the human genomic SOD1 gene encoding the G37R mutation, Gn.SOD1-G37R (line 29), the wild-type sequence, Gn.SOD1-WT (line 76), or Gn.SOD1-H46R/H48Q (line 139) have been previously characterized (Wang et al., 2002b; Wong et al., 1995).

Gene expression analyses

The levels of mRNA expression were determined by Northern blotting as previously described (Thinakaran et al., 1996). Tissues were freshly harvested from animals and extracted with Trizol (Invitrogen, Carlsbad, CA) for total RNA extraction. 5 μg of the total RNAs from each tissue was chromatographed by gel electrophoresis, and then transferred to nylon membrane for Northern blotting. The ^{32}P-labeled DNA probes for the endogenous mouse SOD1 gene were generated by random hexamer priming using a mouse SOD1 cDNA fragment as template. To detect the transgene product, we generated a probe specific for the 3′ untranslated region of PrP mRNAs, which is shared between the endogenous PrP mRNA and the transgene mRNA.

The levels of SOD1 proteins were measured by Western blotting using m/hSOD1 rabbit antiserum which recognizes both the human and the mouse protein (Borchelt et al., 1994). Tissues were homogenized in 1:10 (wt:vol) Phosphate Buffered Saline (PBS, 7.4). Protein concentrations were measured by BCA assay (Pierce, Rockford, IL); 10 μg of protein was used in Western blotting for each sample. Primary antibodies bound to the blot were detected through secondary antibodies linked to horseradish-peroxidase and by enhanced chemiluminescence (PekinElmer Life Sciences, Boston), or with ^{125}I-labeled protein A (Amersham Biosciences, Piscataway, NJ). In the latter case, protein amounts were quantified by PhosphorImager analysis (BioRad, Hercules, CA).

Primary cultures

To isolate astrocytes, cerebral cortices were dissected from postnatal day 1 mice. The meninges and blood vessels were removed from the brain surface before the cerebral cortices were put into DMEM growth medium (with 10% fetal calf serum and Pen/Strep antibiotics) at 4°C. Each brain was minced, digested with 0.25% trypsin, and triturated through pipetting before the resulting cell suspension was plated on poly-D-lysine-coated tissue culture dishes. After passaging, the attached cells twice in the following 15 days, the culture consisted mainly of astrocytes as confirmed by GFAP immunostaining (not shown).

To isolate bone marrow macrophages, the lumen of fractured femurs from 6- to 8-week-old mice was flushed with tissue culture medium. The effluent was diluted in red blood cell lysis buffer and then centrifuged at 500 × *g* for 10 min. The cell pellet was resuspended in tissue culture medium, and ~10^6 cells were plated in a 35-mm Petri dish. The cells were then cultured in medium containing 6 ng/ml murine macrophage-colony stimulating factor (Sigma, St. Louis, MO) for 10 days before further analysis. Differentiation was confirmed by staining with multiple macrophage markers, using Dil-Ac-LDL (Biomedical Technologies, Stoughton MA), anti-CD11b (Caltag Laboratories, Burlingame, CA), and anti-F4/80 (Serotec, Oxford, UK).