Transgenic mice expressing an altered murine superoxide dismutase gene provide an animal model of amyotrophic lateral sclerosis

(motoneuron degeneration/free radicals/age-related disease)

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ABSTRACT  Amyotrophic lateral sclerosis is a progressive neurodegenerative disorder primarily involving motoneurons. A subset of individuals with familial autosomal dominant forms of the disease have mutations of the copper/zinc superoxide dismutase (Cu/Zn SOD, SOD-1) gene, which encodes a ubiquitously expressed enzyme that plays a key role in oxygen free radical scavenging. This observation suggests that altered or reduced SOD-1 activity may play a role in the neurodegenerative process. To explore this possibility further, we have introduced a mutation into the mouse SOD-1 gene that corresponds to one of the changes found in the human gene in familial amyotrophic lateral sclerosis. Integration and expression of this mouse gene in transgenic mice was identified by the presence of a unique restriction enzyme site in the transgene coding sequence generated by introduction of the mutation. We report here that high expression of this altered gene in the central nervous systems of transgenic mice is associated with an age-related rapidly progressive decline of motor function accompanied by degenerative changes of motoneurons within the spinal cord, brain stem, and neocortex. These findings indicate a causative relationship between altered SOD activity and motoneuron degeneration. Moreover, biochemical studies indicate normal levels of total SOD activity in transgenic mouse tissues, results that indicate that the neurodegenerative disorder does not result from a diminution of activity and, as such, represents a dominant “gain of function” mutation.

Amyotrophic lateral sclerosis (ALS) is an age-related, progressive, and fatal neurodegenerative disorder that primarily involves motor neurons (1). Although this disease is variable with regard to age of onset and clinical presentation, it generally affects middle-aged individuals and appears as a motor weakness of the extremities. The disease progresses inexorably, eventually involving cranial nerve motor nuclei and bulbar motor neurons, and patients at the end stages of disease often require substantial medical support to sustain life (1, 2).

Although the majority of ALS cases are sporadic, a subset of affected individuals inherit the disease (3). Rosen et al. (4) observed that a subset of those with autosomal dominantly inherited familial ALS (FALS) harbor mutations of the Cu/Zn superoxide dismutase (SOD-1) gene. These findings suggest a causative relationship between altered or reduced SOD-1 activity and motoneuron degeneration. Subsequent study of SOD-1 activity in erythrocytes of FALS patients carrying mutations at the SOD-1 locus demonstrated reduced SOD activity, leading Deng et al. (5) to propose that it is a dearth of activity that leads to the neurodegenerative syndrome.

Studies of humans with ALS are complicated by the lack of genetic homogeneity or environmental uniformity. Moreover, possible early signs of disease that might appear prior to overt symptoms and could allow preemptive medical intervention are difficult to identify. For these reasons, it is highly desirable to develop an animal model of FALS. The transgenic mouse system (6–8) is well suited to development of such an animal model. Genes introduced into the mouse germ line are often efficiently expressed (9) and can act in a dominant manner to cause diseases that closely resemble the human disorder with respect to phenotype and underlying pathogenetic mechanism (10–12).

We have exploited this system to study the role of altered SOD-1 expression in the pathogenesis of ALS and to attempt to develop an animal model of the disease. We introduced a missense mutation, Gly-86 → Arg, into the fourth exon of a 15-kb mouse genomic clone that we had isolated (13). The same mutation has been observed in the corresponding amino acid residue (position 85) of some patients with FALS (4, 5).

In two lines of mice that produced high levels of transgene mRNA in the central nervous system, motor paralysis developed and was associated with degenerative changes of motoneurons within the spinal cord, brain stem, and neocortex. Biochemical measurements of SOD activity in these animals did not reveal a diminution of activity. These findings establish that altered SOD-1 expression can cause motoneuron degeneration and that this degeneration is not associated with reduced SOD activity. Further, these animals constitute a potentially valuable animal model of ALS.

MATERIALS AND METHODS

Construction of the Altered SOD-1 Gene. A 15-kb genomic clone (13) containing the entire coding sequence of mouse SOD-1 and several kilobases of 3′ and 5′ flanking material was subcloned into the Sal I site of pGEM-5zf(-) (Promega). A 5-kb Xho I–HindIII fragment containing exons 1–4 was subcloned into pGEM-11zf(-) (Promega), released as an EcoRI–HindIII fragment, and subcloned into the plasmid pSELECT-1 (Promega). Site-directed mutagenesis was then performed with the Altered Sites in vitro mutagenesis system (Promega). A 19-base oligonucleotide (5′-GGAGACCTGCAGCAATGTGA-3′) was used to introduce a G → C substitution (underlined) at the first position of codon 86 in exon 4. The sequences of exons 1–4 and the proximal promoter were subsequently confirmed by DNA sequencing. The modified 5-kb Xho I–HindIII fragment was then used to replace the corresponding fragment of the wild-type gene. The completed construct was then excised with Sal I and electroeluted from

Abbreviations: ALS, amyotrophic lateral sclerosis; FALS, familial ALS; SOD, superoxide dismutase; SOD-1, Cu/Zn SOD.

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agarose for microinjection (6, 14) into FVB/N zygotes. PCR primers E3S (21-mer, 5'‐CACCTAAGAAACGTTGCTG-3') and 13S (17-mer, 5'-GACATCTTTGCTTACCC-3') were from sense-strand sequences within exon 3 and intron 3, respectively. Primers E4A (21-mer, 5'-AATGATGAATG-CACCTCTGA-3') and E5A (24-mer, 5'-AGCGCGTC-CCACATTCCAGTC-3') were from antisense sequences within exons 4 and 5.

Identification of Transgenic Mice and Detection of Transgene Expression. Genomic DNA was isolated from tail biopsies. Thirty five cycles of PCR (94°C for 45 sec; 72°C for 1 min; 72°C for 1 min) were performed. PCR products were digested with Fsp I and subjected to electrophoresis on denaturing 8% polyacrylamide gels and stained with ethidium bromide.

For reverse transcriptase–PCR analysis of transgene expression, total RNA was isolated by using the Ultraspec RNA isolation system (Biotex Laboratories, Houston). RNA (500 ng) was reverse-transcribed at 42°C for 1 hr by using primer E5A (see Fig. 1A) and the resulting cDNAs were subjected to 10 cycles of PCR (94°C for 45 sec; 60°C for 1 min; 72°C for 1 min) with primers E3S and E5A. These primers are about 2 kb apart in the genomic intron-containing sequence but are only 233 bases apart in the reverse transcriptase product. An aliquot of the initial PCR mixture was amplified for an additional 30 cycles by using a heminestered primer, E4A, that lies internal to E5A and produces a final product of 141 bp. Transgene transcripts were then detected by Fsp I digestion of the 141-bp product.

Histopathological Studies. Animals were deeply anesthetized with a mixture of ketamine and xylazine and perfused transcardially with 1% ice-cold paraformaldehyde for 1 min followed by 4% (wt/vol) ice-cold paraformaldehyde for 10 min. Brains and spinal cords were rapidly removed, blocked, and cryoprotected in sucrose. Serial 20-μm-thick sections through the brains and spinal cords were cut on a cryostat. One series was mounted and Nissl-stained with cresyl violet, and another parallel series was stained with the Campbell–Switzer silver method (15).

SOD-1 Biochemical Assay. Tissue samples were homogenized in phosphate-buffered saline with a Dounce homogenizer and centrifuged at 12,000 × g for 15 min at 4°C. Protein content of the tissue supernatants was determined using the BCA protein assay (Pierce). Equal amounts of total protein were included from transgenic and corresponding control extracts in assays of SOD-1. SOD-1 activity was measured by the method of Cederbaum et al. (16) with the reaction mixture

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**Fig. 1.** (A) Mutagenesis of the mouse SOD-1 gene. The transgene was constructed by introducing a point mutation at the indicated position (♦) in exon 4. Nucleotides shown in boldface type form a recognition sequence for Fsp I generated by the mutagenesis procedure. Arrows labeled E3S, 13S, E4A, and E5A indicate the positions of the PCR primers used for identification of the transgenic mice and detection of transgene expression by reverse transcriptase–PCR. (B) Identification of transgenic mice. A 143-base PCR product amplified from primers 13S and E4A was digested with Fsp I. The wild-type SOD-1 gene lacks an Fsp I site in this region, whereas the product of the mutant gene, when digested with this enzyme, yields fragments of 86 and 57 bases, as seen in transgenic founders M1, M2, and M3 (lanes 1–3). Other lanes contain nontransgenic control (lane 4), undigested PCR product from founder M1 (lane 5), and PCR with no added template (lane 6). (C) Detection of transgene mRNA in mouse tissues. Reverse transcriptase–PCR products after digestion with Fsp I. The 86- and 55-bp fragments are Fsp I digestion products of the cDNA derived from mRNA containing the mutation. Endogenous mRNA yields an undigested 141-bp product. Lane 14 contains SOD M1 brain mRNA without reverse transcriptase (RT) and lane 15 contains no added RNA.
containing 100 mM KH_2PO_4 (pH 7.5), 0.4 mM hypoxanthine, 0.05 mM cytochrome c, 0.1 mM EDTA, and 0.015–0.03 unit of xanthine oxidase. Cytochrome c reduction was measured at 550 nm for 1 min. All reactions were performed in the presence and absence of 10 mM NaCN, and CN-inhibitable SOD-1 activity was determined by subtraction.

RESULTS

Production of Transgenic Mice and Analysis of Transgene Expression. Fig. 1 shows a map of the 15-kb genomic clone (13) of mouse SOD-1 with the site of the point mutation in exon 4 that results in the Gly-86 → Arg substitution, corresponding to position 85 in human SOD-1 (4, 5). This change also creates a Fsp I restriction site in the DNA, which allows detection of foreign DNA in transgenic mice and RNA transcripts elaborated from the introduced gene (Fig. 1A). Three lines of transgenic mice, M1, M2, and M3, were produced (Fig. 1B). Transgene expression was found in all tissues studied, with widespread high expression in line M1, high expression in brain of line M3, and relatively low expression in brain of line M2 (Fig. 1C).

Mice with High Transgene Expression Exhibit Motoneuron Degeneration. All mice of the low-expressing line M2 appeared phenotypically normal. However, founder M1, a male, was able to breed but, at 3-4 months of age, developed a generalized weakness that progressed within 72 hr to total immobility. All nine transgenic mice of the M1 line thus far studied, including the founder (five females and four males), developed a similar loss of motor function between the ages of 3 and 4 months, with none surviving to the age of 4 months. None of the eight nontransgenic littermates exhibited any abnormalities. Most commonly, a spastic paralysis initially involving the hind limbs was manifest and was associated with profound muscle wasting (Fig. 2A). When these animals were unable to take food and water, they were sacrificed for histological or biochemical analysis (see below). Founder M3, a female, was unable to breed, but at about 100 days of age, developed a similar progressive spastic paralysis of the left hind limb and flank. This animal also developed a generalized loss of motor activity, ceased food and water intake in a manner indistinguishable from affected mice of line M1, and was sacrificed at 110 days of age.

Four animals from line M1 and the founder M3 were sacrificed for histological analysis (Fig. 2) and transgene expression studies of founder M3 (Fig. 1C). Neuropathologic examination of the spinal cord in all transgenic animals revealed moderate to severe degenerative changes within the ventral horns (Fig. 2B and C). In the most severe case studied, there was a pronounced loss of large spinal motoneurons compared to controls, with remaining motoneurons exhibiting pyknosis and karyorrhexis. Several ghost cells were also seen (Fig. 2D and E). Silver staining showed numerous dystrophic neurites in the ventral horn gray matter, large and small argyrophilic perikarya, and swollen fragmented processes (Fig. 2B and C). A few dystrophic neurites were observed in the dorsal horn gray matter, whereas no argyrophilic perikarya were detected. In animals with less severe pathology, fewer abnormal neurites and perikarya were found in the spinal cord. The severity of the alterations was consistent in different spinal cord levels within each animal. Similar spinal cord pathology was seen in founder M3. Although we could not perpetuate line M3, the appearance of a similar motor disorder and histopathological changes in two transgenic lines, in association with high levels of transgene mRNA in brain (Fig. 1C), provides compelling evidence that the abnormalities in animals of both lines result from transgene expression.

Within the brain stem and neocortex, degenerative changes were seen in motor components of cranial nerve nuclei (Fig. 2 G and H). Degeneration of a few cortical motoneurons in layer V was also observed (Fig. 2F). Some degenerative changes were also found in the hypothalamus, deep layers of the superior colliculus, deep cerebellar nuclei, basal ganglia, and thalamus.

We have also generated three lines of transgenic mice with the wild-type mouse SOD-1 gene. More than 100 of these animals have been observed for more than 6 months, and no abnormalities have been noted.

Transgenic Mice with Neurodegeneration Have Normal Levels of SOD Activity. Studies of SOD-1 biochemical activity in FALS patients, including some individuals with the Gly → Arg mutation at position 85 of SOD-1, revealed a substantial diminution of SOD-1 activity, findings that have led to the

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**Fig. 2.** Phenotypic and histopathological changes in transgenic mice from line M1. (A) Transgenic mouse. Spastic paralysis of the hindlimbs and right forelimb with associated muscle wasting is apparent. (B and C) Silver-stained sections of the lumbar spinal cord in control (B) and transgenic (C) animals. Extensive degeneration of the ventral horn gray matter is present in the transgenic sample (arrows), whereas the dorsal horns (asterisks) are relatively unaffected. Corresponding tissues of the control are normal. (D and E) High magnification of Nissl-stained sections of the ventral horn gray matter in control (D) and transgenic (E) animals. There is severe loss of large motoneurons in the transgenic mouse, with typical cellular alterations such as ghost cells and pyknosis (arrows) (E). (F) Silver-impregnated perikaryon and proximal apical dendrite of a degenerating cortical motoneuron in a transgenic animal. Pial surface is to the right. (G and H) Silver-stained sections through thepons at the level of the motor nucleus of the trigeminal nerve in control (G) and transgenic (H) animals. Note the severe pathological changes of the motor nucleus in the transgenic mouse (arrows) with numerous argyrophilic perikarya and swollen dystrophic processes, whereas the principal sensory nucleus of the trigeminal nerve (asterisks) is unaffected. (Bar in H: B and C, 200 μm; D and E, 25 μm; F, 17.5 μm; G and H, 30 μm.)
hypothesis that reduced SOD-1 activity is responsible for neuronal degeneration (5). We determined SOD-1 activity in five tissues of four M1 transgenic mice (16) relative to age- and sex-matched controls, in the same manner as others have done in studies of transgenic mice with the normal human SOD-1 gene (17). As shown in Table 1, no marked differences in activity relative to controls were observed. In two animals near death at the time of sacrifice, some diminution of activity in liver was noted, but this probably relates to the poor nutritional status of these animals, as younger transgenic mice of this line exhibit normal hepatic SOD-1 activity. Thus, in our animals, motoneuron degeneration is not associated with reduced SOD-1 activity.

DISCUSSION

Our findings demonstrate an invariant association between high expression of a mutated murine SOD-1 gene and development of an age-related motor disorder accompanied by degenerative changes of neurons throughout the motor system. These observations have been made in two mouse lines with different integration sites for the transgene, thus rendering prohibitively unlikely the possibility that the phenotype is the result of a mutation at the loci of transgene insertion. Because the sequence change introduced into the gene corresponds to a mutation of the human SOD-1 gene found in some patients with FALS, the findings have significant implications for efforts to develop an animal model of ALS and for the study of oxidative stress and neuronal cell loss.

In humans with FALS who harbor mutations at the SOD-1 locus, many genetic and environmental variables complicate analysis of the relationship between the mutations found and the appearance of this disease. The transgenic mice produced are of the highly inbred FVB/N strain (18) and are housed under uniform conditions. The observation that animals transgenic for the altered SOD-1 gene develop motoneuron degeneration and the correlation between such degeneration and high-level expression of the mutant gene in the central nervous system provide compelling evidence that altered SOD-1 activity causes the neurodegenerative changes observed. If it is assumed that the pathogenetic mechanisms by which alterations of SOD-1 cause motoneuron degeneration are the same in these mice and in humans with FALS, several important inferences can be drawn concerning FALS as a disease entity.

First, biochemical studies on our animals are not consistent with the notion (5) that reduced SOD-1 activity is responsible for the neurodegeneration. SOD-1 activity in the transgenic mice is not significantly different from that of controls (Table 1). While these experiments do not rule out the possibility that motoneurons undergo a selective reduction of SOD-1 activity as a result of transgene expression, we consider this explanation unlikely. We have likewise not formally ruled out the possibility that the mutation may greatly reduce stability of the enzyme such that activity in vivo may be different from that measured in vitro. Consistent with this possibility, we have not observed transgene-specific electrophoretic SOD variants in transgenic tissue extracts by using a variety of electrophoresis systems including isoelectric focusing (data not shown).

Although we have not formally proven that SOD activity is not reduced in motoneurons in vivo, it is noteworthy that while the Gly 85 → Arg mutation significantly reduces SOD-1 activity in humans, many of the other FALS mutations do not substantially alter activity (5), and partial or complete monosomy 21, which reduces SOD-1 activity, is not associated with selective loss of motoneurons (19, 20). We accordingly believe the most logical explanation for the findings is that the mutant gene employed in our experiments encodes an enzyme with low SOD activity, and thus, expression of the altered enzyme does not significantly affect overall activity when added to the genome in the presence of two wild-type parental genes. However, activity of the altered enzyme may generate other free radicals that are toxic, as has been theorized by others (21). As such, these SOD-1 mutations may be viewed as “gain of function” rather than “loss of function” mutations.

Although the initial signs of a motor disorder differed between animals of the M1 line, with some animals developing a flaccid paralysis and others a spastic paralysis involving either the forelimbs or hindlimbs (Fig. 24), the age of onset was very predictable and the progression of the disease was very rapid: all affected animals were normal at the age of 3 months but dead by 4 months, and the disorder progressed from a mild gait abnormality to total paralysis within a 5-day period in all cases. These observations are consistent with the hypothesis that the time required to accumulate sufficient oxidative damage to cause neuronal demise is very similar and that in these mice cells throughout the motor system are synchronously brought to the threshold of degeneration before signs of disease first appear. The subgroups of cells that first cross that threshold vary between individuals, thus accounting for the variable presentation of the disorder. The rapid progression of paralysis indicates that remaining affected motoneurons die soon thereafter. If this interpretation is correct, then we may infer that clinical heterogeneity of ALS need not be attributable to multiple pathogenetic mechanisms for the disease, but rather, to genetic and environmental variables that contribute to relatively asynchronous neuronal degeneration in affected humans. If motoneurons in the transgenic mice are uniformly affected, studies of early signs of damage may be feasible.

Because the cause of neurodegeneration in these mice appears identical to at least a subset of humans with FALS, these animals should provide a useful model of the disease. The short lifespan of the mouse allows for efficient investigation of age-related neurodegenerative processes that require many years to develop in primates. Other possible transgenic mouse models of ALS have been produced by overexpression neurofilament genes (22), though it is not yet clear that the human disease is caused by this mechanism. As a possibly highly representative model of FALS, the animals have important potential not only for identifying early events in the neurodegenerative process but also for attempting preemptive therapy.

Note Added in Proof. Since submission of this manuscript, another publication has reported similar findings in transgenic mice after insertion of a human SOD gene with a mutation at amino acid position 93, a mutation also associated with FALS (23).
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