Transgenic Small Interfering RNA Halts Amyotrophic Lateral Sclerosis in a Mouse Model*

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RNA interference is the process of sequence-specific, post-transcriptional gene silencing, initiated by double-stranded RNA. RNA interference is a multistep process that involves generation of 21–23-nucleotide small interfering RNA (siRNA), resulting in degradation of homologous RNA. One rational approach to therapy using siRNA is to eliminate the aberrant protein encoded by mutant alleles in dominantly inherited diseases.

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease characterized by the degeneration of motor neurons in the central nervous system. Although most cases of ALS are sporadic, 5–10% of ALS cases are familial, and of these, ~20% are due to missense point mutations in the gene encoding copper/zinc superoxide dismutase (SOD1) (1). Recent studies using transgenic (Tg) mice and cell culture models of ALS with SOD1 mutations have indicated that SOD1 mutations induce the disease by their toxic properties, not by a loss of SOD1 activity (2). Therefore, inhibition of mutated allele expression is expected to provide a direct approach to therapy for this type of familial ALS. In cultured cells, siRNA can effectively inhibit the production of mutant proteins in various neurodegenerative diseases including ALS (3). Furthermore, virus-mediated siRNA delivered by direct injection of viral vectors to the brain or muscle delays phenotypic expression in Tg mice in vivo (4–7). However, it has not been proved in principle whether inhibition of mutant genes with siRNA can truly stop dominantly inherited diseases. The most difficult problem in in vivo therapy with siRNA is that there is no sophisticated method of delivering siRNA throughout the central nervous system. Therefore, to answer this question, as a first step, we tried to make siRNA Tg mice in which siRNA was ubiquitously expressed in the brain, and we then crossed these siRNA Tg mice with SOD1G93A Tg mice to efficiently deliver siRNA throughout the central nervous system. Moreover, we utilized modified short hairpin RNA (shRNA), which has mismatch alternations within the sense strand, to make Tg mice. This method was able to enhance the genetic stability of the shRNA expression cassette in the genome over generations.

MATERIALS AND METHODS

Construction of Anti-SOD1 shRNA Expression Vector—we generated an anti-SOD1 shRNA cassette as reported previously (3). We inserted the anti-SOD1 shRNA cassette immediately downstream of the human U6 promoter in pUC19 (Takara, Tokyo, Japan), with a PGK-neo-poly(A) cassette (Fig. 1A). Three G → A alternations were introduced (denoted by asterisks below) in the sense strand: 5′-GGUGG*AAUUG*AAAGAAGUAC-3′ (Fig. 1B). This sequence was a good and common siRNA target region in both human and mouse SOD1 mRNAs. To select this target site, we performed a BLAST similarity search to minimize off-target effects.

Generation of Anti-SOD1 siRNA Tg Mice—To produce anti-SOD1 siRNA Tg mice, the anti-SOD1 shRNA expression vector was introduced into 129/Sv embryonic stem (ES) cells (Chemicon, Temecula, CA) by electroporation, and individual stable integrants were tested for expression of SOD1 protein by Western blot analysis. ES cell clones that exhibited greatly decreased SOD1 expression were injected into C57BL/6 blastocysts (CLEA Japan, Tokyo, Japan), and the resulting chimeric male mice were mated with C57BL/6 females. The offspring, in which germline transmission was determined by the following PCR method, were referred to as anti-SOD1 siRNA Tg mice.

Double Tg mice were generated by crossing SOD1G93A Tg mice (G1H line from Jackson Laboratories, backcrossed to C57BL/6 mice) with anti-SOD1 siRNA Tg mice. Genotypes of these mice were determined...
by PCR analysis of tail DNA. PCR was carried out using the following primer sets: 5'-CTTGGGTAGTTTGCAG-3' and 5'-CAGGAAA-CAGCTATGAC-3' for anti-SOD1 siRNA Tg mice and 5'-CATCAG-CCCTAAATCCATCTGA-3' and 5'-CGGACTAACAATCGAAGTGA-3' for SOD1G93A Tg mice. The mice were maintained under pathogen-free conditions and handled in accordance with the Guidelines for Animal Experimentation of the Institute for Advanced Technology of Kinki University and of Tokyo Medical and Dental University.

Northern Blot Analysis—Mice were deeply anesthetized with pentobarbital sodium, sacrificed, and perfused with cold phosphate-buffered saline. Total RNA was extracted from the brain and spinal cord by using TRIzol (Invitrogen). Total RNA (20 μg) was fractionated on a 1% formaldehyde agarose gel and transferred to a Nytran membrane (Schleicher & Schuell). The lower part of the membrane was hybridized with a purified PCR fragment, corresponding to mouse SOD1 cDNA (bases 15–495); it was labeled with fluorescein by using a Gene Images random-prime labeling kit (Amersham Biosciences). The upper part of the membrane was hybridized with a probe specific for β-actin. The signals were visualized with a Gene Images CDP-star detection kit (Amersham Biosciences). For detection of small RNA, total RNA (25 μg) was separated by electrophoresis on a 14% polyacrylamide-urea gel and transferred to a Hybond-N+ membrane (Amersham Biosciences). The blot was hybridized with a probe of the non-mutated sense sequence of shRNA, which was labeled with fluorescein by using a Gene Images 3'-Oligolabeling kit (Amersham Biosciences) and visualized as mentioned above.

Western Blot Analysis—ES cell lysates were prepared with radioimmune precipitation buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS). Protein samples were extracted from tails, brains, and spinal cords and homogenized in buffer containing 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, and 1 mM phenylmethylsulfonyl fluoride. Equal amounts of extracted protein were mixed with Laemmli sample buffer, denatured, and separated on 15% SDS-PAGE. After transfer to a polyvinylidene difluoride membrane (Bio-Rad), blots were probed with anti-SOD1 polyclonal antibody S-100 (1:7000, StressGen Biotechnologies, Victoria, British Columbia, Canada) or anti-β-tubulin monoclonal antibody (1:500, BD Biosciences) followed by the relevant horseradish peroxidase-conjugated immunoglobulin (Amersham Biosciences). Immunoblots were detected using ECL reagent (Amersham Biosciences).

Immunohistochemical and Histopathological Analyses—The lumbar segments of the spinal cords were removed and fixed in 4% paraformaldehyde in phosphate-buffered saline, pH 7.4. They were cryoprotected with sucrose protected with sucrose solution and frozen in Tissue-Tek O.C.T. compound (Sakura Fine-technical Co., Tokyo, Japan). For immunohistochemistry, sections (10 μm thick) of the spinal cord at the level of the third lumbar (L3) vertebra from anti-SOD1 siRNA Tg mice and wild-type littermates were mounted onto the same gelatin-coated slide and incubated with anti-SOD1 polyclonal
antibody S-100 (1:1000, StressGen Biotechnologies). Staining was visualized by diaminobenzidine. For histopathological examination of the tissues of double Tg mice, SOD1<sup>G93A</sup> Tg mice, and wild-type littermates, sections 10 μm thick were stained with hematoxylin and eosin.

L3 ventral roots were taken from the spinal cord and fixed in phosphate-buffered 2.5% glutaraldehyde, postfixed in 1% osmic acid, and then embedded in Epon. Toluidine blue-stained semi-thin transverse sections of these materials were used for evaluation of the density and size distribution of myelinated fibers.

**Determination of Disease Onset and Progression**—We compared the motor functions of double Tg mice with those of SOD1<sup>G93A</sup> Tg mice and wild-type littermates by using a rotating rod apparatus (accelerating model, Ugo Basile Biological Research Apparatus, Varese, Italy). The mice were placed on the rod for four trials. Each trial lasted a maximum of 4 min, during which the rotating rod underwent linear acceleration from 4 to 33 rpm over 4 min. Disease onset was determined by the presence of hindlimb paresis on walking. Mortality was scored as date of death or inability of the mouse to right itself within 30 s of being placed on its side.

**FIGURE 3.** The silencing effect of siRNA is stable. SOD1 protein levels with aging (left) and from generation to generation (right) are shown. F1 and F3 mice were examined at 1 month (mo) of age. Values are the ratios to age-matched wild-type (Wt) littermates (mean and S.E.). n = 3 for each group. ns = not significant (p > 0.05, Student’s t test).

**FIGURE 4.** Double Tg mice had a marked reduction in the amount of G93A SOD1 protein in the spinal cord and did not show the ALS phenotype. **A,** detection of the antisense strand of siRNA in the spinal cord on Northern blot analysis. nt, nucleotides. **B,** levels of both mutant G93A SOD1 and mouse SOD1 proteins were similarly reduced in the spinal cords of double Tg mice on Western blot analysis. The level of G93A SOD1 protein in double Tg mice was lower than that of the low copy strain of SOD1<sup>G93A</sup> Tg mice. Lane 1, SOD1<sup>G93A</sup> Tg mouse; lane 2, low copy strain of SOD1<sup>G93A</sup> Tg mouse; lane 3, double Tg mouse; lane 4, wild-type mouse. **C,** this SOD1<sup>G93A</sup> Tg mouse at 130 days of age showed paralysis of both hindlimbs. In contrast, the double Tg mouse at the same age walked well. **D,** cumulative probabilities of onset of disease signs (left) and survival (right). There was a significant increase in the life span of the double Tg mice (n = 6; closed circles) compared with the SOD1<sup>G93A</sup> Tg mice (n = 23; open circles). **E,** performances on the accelerating rotating rod apparatus. **F,** growth curves of female mice. Values are means and S.E.
RESULTS

Anti-SOD1 siRNA Tg Mice—We obtained 3 of 50 G418-resistant ES cell clones that showed an ~80% reduction in the level of endogenous SOD1 protein by Western blot analysis (Fig. 2A). Each ES cell clone was injected into C57BL/6 blastocysts, and chimeric male mice with high levels of ES cell descendants were obtained. These chimeras were outcrossed, and germline transmission of the shRNA was noted in numerous F1 progeny from one ES line (12/35) on PCR analysis. In the brains of anti-SOD1 siRNA Tg mice, expression of siRNA was clearly detected (Fig. 2B), and mouse SOD1 mRNA was strikingly reduced on Northern blot analysis (Fig. 2C). The level of SOD1 protein was also suppressed by about 80% on Western blot analysis (Fig. 2D). Anti-SOD1 siRNA Tg mice did not show any obvious phenotype such as growth retardation or motor signs, with the exception of infertility in females. In immunohistochemical analysis, the SOD1 immunoreactivity of both the gray and the white matter of the spinal cord in the anti-SOD1 siRNA Tg mice was much lower than that in the wild-type littermates. In the anterior horn of the spinal cord in anti-SOD1 siRNA Tg mice, the SOD1 immunoreactivity was reduced dominantly in the non-neuronal cells and neuropils (Fig. 2E).

The Silencing Effect of siRNA Is Stable with Age and through the F3 Generation—To analyze changes in SOD1 protein levels with age and through the generations, we examined SOD1 protein levels in the tails of anti-SOD1 siRNA Tg mice and age-matched wild-type littermates by Western blot analysis. There was no obvious decrease in the effect of siRNA on knockdown of SOD1 production at 1 and 11 months old or in F1 and F3 mice (Fig. 3).

Mutant G93A SOD1 Protein Production Is Decreased in Double Tg Mice—By crossing anti-SOD1 siRNA Tg mice with SOD1G93A Tg mice, we obtained six double Tg mice and 26 SOD1G93A Tg mice. In the spinal cords of the double Tg mice, we clearly detected the expression of siRNA (Fig. 4A). Levels of both mutant human G93A SOD1 protein and mouse wild-type SOD1 protein in the spinal cords of the double Tg mice were similarly reduced. The percentage of reduction of mutant G93A SOD1 in the spinal cord was estimated to be about 80%. The level of mutant G93A SOD1 protein in the double Tg mice was about half that in the low copy strain of SOD1G93A Tg mice (G1L/ from Jackson Laboratories, backcrossed to C57BL/6 mice) in which disease onset occurred at 280 days of age (Fig. 4B).

Phenotype of Double Tg Mice Is Normal—SOD1G93A Tg mice showed the first signs of motor deficits at a mean age of 127.3 ± 1.2 days. All of these mice then deteriorated progressively, showing a lack of mobility, failure to groom their fur, hindlimb dysfunction, breathing difficulties, and muscle atrophy. All SOD1G93A Tg mice were dead by 157 days of age (Fig. 4, C and D). In contrast, double Tg mice appeared normal and grew up similarly to wild-type littermates. Their motor performance on the rotating rod test did not differ from that of wild-type littermates over the entire 300-day duration of the experiment (Fig. 4E). The weights of SOD1G93A Tg mice declined just before the onset of disease, but double Tg mice did not lose weight (Fig. 4F). The online supplemental movie dramatically shows that the transgenic anti-SOD1 siRNA completely prevented the development of the ALS phenotype seen in SOD1G93A Tg mice (see Supplemental Movie).

Histological analysis of the spinal cord was performed at the end of disease in SOD1G93A Tg mice and at 6 months of age in double Tg mice and the age-matched wild-type littermates. In the SOD1G93A Tg mice,
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the spinal cord at L3 showed a severe loss of motor neurons with an increase in the numbers of astrocytes (see Supplemental Figure), and myelinated axons in the L3 ventral root were atrophic and less dense. In contrast, the spinal motor neurons and axons in the double Tg mice appeared normal (Fig. 5, A and B).

DISCUSSION

One serious problem in using shRNA to generate Tg mice is that mutations can occur within the hairpin region of the shRNA sequence during replication, leading to a reduction in silencing efficiency with age and over generations. In fact, ~20% of our constructs without mismatch alternation were mutated within the hairpin region of the constructs upon introduction into Escherichia coli (8), and some anti-green fluorescent protein Tg mice lost the knockdown effect in the F1 generation, even with expression of siRNA (9). We previously showed that mismatch alternation of a few nucleotides in only the sense strand prevented mutation during replication without reducing the silencing effect (8). Thus, we introduced three mismatch alternations in the sense strand, and all of our anti-SOD1 Tg mice showed no decrease in siRNA effect over four generations. The mismatch alternations in the sense strand of our shRNA might have prevented a decrease in the siRNA effect in vivo. In view of these results, we think that crossing of siRNA Tg mice could be a useful strategy for analyzing the effect of knockdown of the gene of interest on the phenotype of the crossed mice.

Our results showed that development of the ALS phenotype in SOD1<sup>G93A</sup> Tg mice was completely suppressed by crossing with anti-SOD1 siRNA Tg mice. In our double Tg mice, siRNA overexpressed against the SOD1 gene in anti-SOD1 siRNA Tg mice cleared the mRNA of G93A SOD1 expressed in the crossed mice. We consider that prevention of development of the ALS phenotype in the double Tg mice was caused by the knockdown effect on SOD1 protein production. The mouse wild-type SOD1 gene was similarly inhibited by the siRNA, but elimination of wild-type SOD1 has been reported to have no effect on the mutant SOD1-mediated ALS phenotype (10). An off-target effect of the siRNA on other unidentified mouse genes is also improbable. This is because 1) a BLAST search for our shRNA sequence showed no match in other areas of the mouse genome and 2) the infertility observed in female anti-SOD1 siRNA Tg mice has also been reported in mice with knock-out of the SOD1 gene (11). Moreover, a close relationship between the copy number of G93A SOD1 and time of onset of the ALS phenotype is known to occur in SOD1<sup>G93A</sup> Tg mice (12). More recently, direct injection of an siRNA-expressing viral vector into the spinal cord (5) or skeletal muscles (6, 7) is reported to reduce the severity of the ALS phenotype in SOD1<sup>G93A</sup> Tg mice. Although viral vector-mediated siRNA delayed the onset of disease or the decrease in grip strength, none of these vector-mediated siRNAs could prevent the disease. In contrast, our double Tg mice did not show any motor dysfunction at 300 days and were expected to remain free of signs of disease at 2 years of age, the time at which disease onset has been predicted from the rate of reduction in the amount of mutant SOD1 in the spinal cord (12). Most likely, this difference can be explained by the possibility that our transgenic siRNA had a greater knockdown effect than did the viral vector-mediated siRNA. Alternatively, reduction in the amount of mutant SOD1 in non-neuronal cells as well as neuronal cells in our double Tg mice might have contributed to the better outcome; the effects of siRNA were limited to the motoneurons when viral vectors were injected into the skeletal muscles (6, 7). There are several lines of evidence that production of mutant SOD1 in both neuronal and non-neuronal cells is critical in the mechanism of the disease (13–16).

Our findings clearly demonstrated that siRNA halted familial ALS by silencing the mutant gene. If a non-invasive method of delivery of siRNA to both neuronal and non-neuronal cells throughout the central nervous system can be developed, the concept of truly overcoming these autosomal dominantly inherited neurodegenerative diseases will no longer be an impossibility.

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