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Establishing a standardized therapeutic testing protocol for spinal muscular atrophy

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Several mice models have been created for spinal muscular atrophy (SMA); however, there is still no standard preclinical testing system for the disease. We previously generated type III-specific SMA model mice, which might be suitable for use as a preclinical therapeutic testing system for SMA. To establish such a system and test its applicability, we first created a testing protocol and then applied it as a means to investigate the use of valproic acid (VPA) as a possible treatment for SMA. These SMA mice revealed tail/ear/foot deformity, muscle atrophy, poorer motor performances, smaller compound muscle action potential and lower spinal motoneuron density at the age of 9 to 12 months in comparison with age-matched wild-type littermate mice. In addition, VPA attenuates motoneuron death, increases spinal SMN protein level and partially normalizes motor function in SMA mice. These results suggest that the testing protocol developed here is well suited for use as a standardized preclinical therapeutic testing system for SMA.

Keywords: Animal model; Motoneuron disease; Neuronal degeneration; Phenotype; Spinal cord; Spinal muscular atrophy; Valproic acid

Introduction

Spinal muscular atrophy (SMA) is characterized by the degeneration of spinal cord motoneurons, and it is associated with muscle paralysis and atrophy. Childhood SMA exhibits an autosomal recessive pattern of inheritance with an incidence of 1 in 6000–10,000 newborns (Czeizel and Hamula, 1989). Based on age of onset of symptoms and achievement of motor milestones, SMA has been subdivided into three clinical types (Munsat, 1991; Talbot and Davies, 2001). Due to gene deletions, mutations or conversions, the telomeric copy of the *survival of motor neuron* gene (*SMN1* [MIM 600354]) is abnormal in about 96% of all SMA patients (Wirth, 2000). The centromeric *SMN* gene (*SMN2* [MIM 601627]) is still present in all of these SMA patients but is not able to compensate for the *SMN1* gene defect (Wirth, 2000).

No curative treatment is available so far for SMA. Several mice models for SMA have been created through different methods (Monani et al., 2000; Hsieh-Li et al., 2000; Frugier et al., 2000; Le et al., 2005; Jablonka et al., 2000), and many potential treatments have been assessed in these model mice to test therapeutic effects *in vivo* (Chang et al., 2001; Lesbordes et al., 2003; Haddad et al., 2003; Azzouz et al., 2004). But because of the disadvantage of short survival time (less than 40 days), heterogeneous phenotype or the non-homologous genetic situation compared to human SMA in these mice models, there is still no standard preclinical therapeutic testing system for SMA. We previously generated three different types of SMA-like mice by the mouse *Smn* knockout–human *SMN2* transgenic method (Hsieh-Li et al., 2000), however, the heterogeneous disease severity within litters made the study design difficult. After purifying the genetic background of our previous model mice (Hsieh-Li et al., 2000), we have obtained a congenic mouse strain, which can consistently breed type III-specific SMA mice (the mildest form of SMA) (Tsai et al., unpublished observations). These type III SMA mice have a lifespan of longer than half a year, offering a chance to become a good therapeutic testing system; however, their phenotypes have not been investigated in detail. The first aim of this study was to evaluate the use of these type III SMA mice as a preclinical therapeutic testing system. In order to do this, we firstly characterized our type III SMA by a series of examinations including morphological, motor functional, electrophysiological and pathological studies. We then investigated the therapeutic effects of valproic acid (VPA) on type III SMA mice to test its applicability. We previously confirmed the benefits of sodium butyrate treatment in our SMA model mice (Chang et al., 2001), however, its adverse effects in humans preclude clinical use (Monneret, 2005). VPA, being a histone deacetylase inhibitor similar to sodium butyrate, increased SMN protein levels in recent

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studies using cells from SMA patients (Brichta et al., 2003; Sumner et al., 2003). Here, we tried to apply VPA to our SMA mice.

Materials and methods

Mice

Heterozygous mouse *Smn* knockout–human *SMN2* transgenic mice (Hsieh-Li et al., 2000) ($Smn^{+/-}SMN2^{+/-}$) were backcrossed with C57BL/6J inbred mice (National Laboratory Animal Center, Taiwan) to purify the genetic background. After crossing for 6 generations, the offspring $Smn^{+/-}$ mice with or without *SMN2* transgenes were intercrossed to generate SMA mice ($Smn^{-/-}$ $SMN2^{+/-}$). All these SMA mice survived longer than 6 months and were classified as type III SMA mice (Tsai et al., unpublished observations). Because these wild-type SMA mice usually die between 9 months and 15 months old, we analyzed them before the age of a year.

In a SMA characterization study, 20 type III SMA mice ($Smn^{-/-}$ $SMN2^{+/-}$) and 20 wild-type mice ($Smn^{+/+}$) were generated through intercrossing heterozygous knockout mice with or without transgene (No. 566 or 576). These 20 SMA mice and 20 wild-type mice were subdivided into four subgroups, according to their evaluated age: 3, 6, 9 and 12 months old ($n=5$ in each subgroup). Each mouse underwent morphological, motor functional, electrophysiological and pathological studies. For the VPA application study, 8 SMA mice ($Smn^{-/-}SMN2^{+/-}$) and 8 heterozygous *Smn* knockout mice ($Smn^{+/-}$) were obtained by intercrossing a type III SMA mouse (No. S555) with its $Smn^{+/-}$ littermate mouse. VPA (valproic acid sodium salt, P4543, Sigma) at 0.2 mg/ml was added to the daily drinking water to treat four out of eight SMA ($Smn^{-/-}$ $SMN2^{+/-}$) and four out of eight heterozygous mice ($Smn^{+/-}$) from the age of 1 month after genotyping. At 9 months old, the 16 mice with or without drug therapy were subjected to the morphological, functional and electrophysiological tests and then sacrificed for molecular and pathological studies.

Mice were supplied with sterile water and rodent pellets ad libitum and were under the care of the animal facility of the Institute of Molecular Biology, Academia Sinica, Taiwan. All procedures were approved by the Academia Sinica Animal Care and Use Committee, Master Protocol # RMiRbIMBLH2001132.

Genotyping

We analyzed the mouse genotypes of *Smn* knockout and *SMN2* transgene by polymerase chain reaction (PCR) analysis. Genomic DNA samples from mouse tails were prepared as previously described (Hsieh-Li et al., 2000). The three specific PCR primers (S1, S2 and H1) for detection of the wild-type and mutant *Smn* alleles and the primer pair (3F and 3B) used to identify the *SMN2* transgenes have been previously described (Hsieh-Li et al., 2000).

Motor function tests

We used four sets of functional tests to evaluate the motor function of mice similar to those used in previous reports, including a tilting test (Kuhn and Wrathall, 1998), a wheel running test (Manabe et al., 2002), a RotaRod maintenance test (Li et al., 2000; Kaspar et al., 2003) and a landing foot spread test (Edwards and Parker, 1997).

In the tilting test, the mice were placed on a clean, dry wood platform, which could be tilted from 0° to 90°. The highest degree of inclination at which the mice could hold on for 5 s was recorded. In the wheel running test, the mice were placed in a circular cage with a diameter of 13 cm and a width of 4.5 cm. After brief training, the number of cycles run by the mouse per minute was counted while the mouse ran voluntarily in an unilateral direction. We averaged the running circles of the best two out of three consecutive tests for each mouse. For the RotaRod maintenance test, the rolling rate of the transverse rod (rod, diameter 3.5 cm, Acceler RotaRod 7650, UGO BASILE, Varese, Italy) was accelerated with a speed of 8 cycle/min. After brief training, the mice were placed on the rotating rod with continuous acceleration of rotating speed. The time each mouse remained on the rod was registered automatically. We stopped the test if the mouse could remain on the rod for more than 300 s. Results of the best two out of five individual tests on each mouse were averaged. In the landing foot spread test, each mouse was held in a horizontal position about 30 cm above the bench with the dorsal up. The mouse was then dropped, and the position of the pre-inked fourth digit of each hind limb on landing was marked. We measured the distance between the two marks, and the mean distance in the best two out of five consecutive tests was calculated for each mouse.

Electrophysiological study

Mice were anesthetized intraperitoneally with 60 mg/kg of pentobarbital. We exposed bilateral sciatic nerves of mice around the sciatic notch. Supramaximal square pulses, of 0.1 ms duration (S88, Grass-Telefactor, RI), were delivered through a stimulus isolation unit (SIU5, Grass-Telefactor, RI), a constant current unit (CCU1A, Grass-Telefactor, RI), and finally a pair of bipolar electrodes with the cathode placed distally on the sciatic nerve at the sciatic notch level. An active recording bipolar needle electrode (length of 5 mm with the two needles 1 mm apart) was inserted in the medial part of the gastrocnemius muscle. The signal was amplified (50 times), and the myoelectric artifacts were bandpass filtered (30 Hz–3 kHz) by a preamplifier (P511 AC, Grass-Telefactor, RI). The recorded signal from the muscle and the stimulating signal directly from the stimulator were analyzed by computer system (MP30, BIOPAC system, CA). A biphasic waveform indicated recording at the motor point as compound muscle action potential (Kennel et al., 1996). We recorded the peak-to-peak amplitude of the biphasic wave. The results from the left and the right gastrocnemius muscles were measured and averaged. Throughout the procedure, mice were kept under a heating lamp to maintain a physiological temperature.

Pathological study

After sacrificing mice, we rapidly removed and fixed lumbar spinal cords in 10% neutral-buffered formalin (Fisher Scientific, PA) overnight at room temperature, serial dehydrated and then embedded them in paraffin (Tissue-Tek VIP, Sakura, CA). Spinal cords were cut transversely at a thickness of 7 μm throughout the level of L4–5 spinal cord, and every seventh section was stained with H&E as previously described (Hsieh-Li et al., 2000). The samples were observed, and images were captured digitally with a light microscope (Leica DM RA, Leica Microsystems, Germany). Neurons were identified according to their pyramidal shape, large nucleus, prominent nucleolus and presence of Nissl body. The cell